

ANNUAL REVIEW OF BIOCHEMISTRY

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PREFACE

The preparation of a preface to each succeeding volume of the *Review* is somewhat more than a routine task of which we may dutifully but, nevertheless, lightly dispose. We choose to regard it as an occasion to report upon changes in editorial policy, but even more as a pleasurable opportunity whereby we may join with our readers in paying tribute to the writers of these reviews. Their task has called for the exercise of judgment, discrimination, and forbearance under circumstances of unusual difficulty. Year by year severe restrictions have had to be imposed upon the lengths of the various reviews. Many substantial works have had to be placed aside—a circumstance which is trying to an author, if not vexatious.

The tide of papers of biochemical interest continues to rise but only a fraction can be selected for review. Over 10,000 abstracts of papers in biological chemistry appeared in *Chemical Abstracts* in 1936. The science has lost none of its youthful vigor. Even a casual reader cannot fail to be impressed by its lively activity, by the enthusiasm with which new regions are explored, and by the wealth of discovery which follows upon fundamental investigations in scattered fields.

Perhaps the only change in the composition of the *Review* to which we need refer is the inclusion of a subject index. A number of circumstances, now past, caused us to doubt the feasibility of including subject indexes in the earlier volumes, but we trust that the preparation of a cumulative index at a later date may remedy this deficiency.

The policy of including a few reviews on subjects of a timely nature is to be continued. We are glad to publish in the present volume two such reviews—one on the applications of microchemistry to biochemical analysis and the other on the biochemistry of fish. Volume VII will include reviews on insecticides and fungicides of plant origin and on chemotherapy.

To those who have been good enough to make suggestions in respect to authorship and topics appropriate for review, who have reminded us of corrections calling for notation among the errata, who have furnished the reviewers with reprints of their published works, or who have assisted in other ways, we express our sincere thanks.

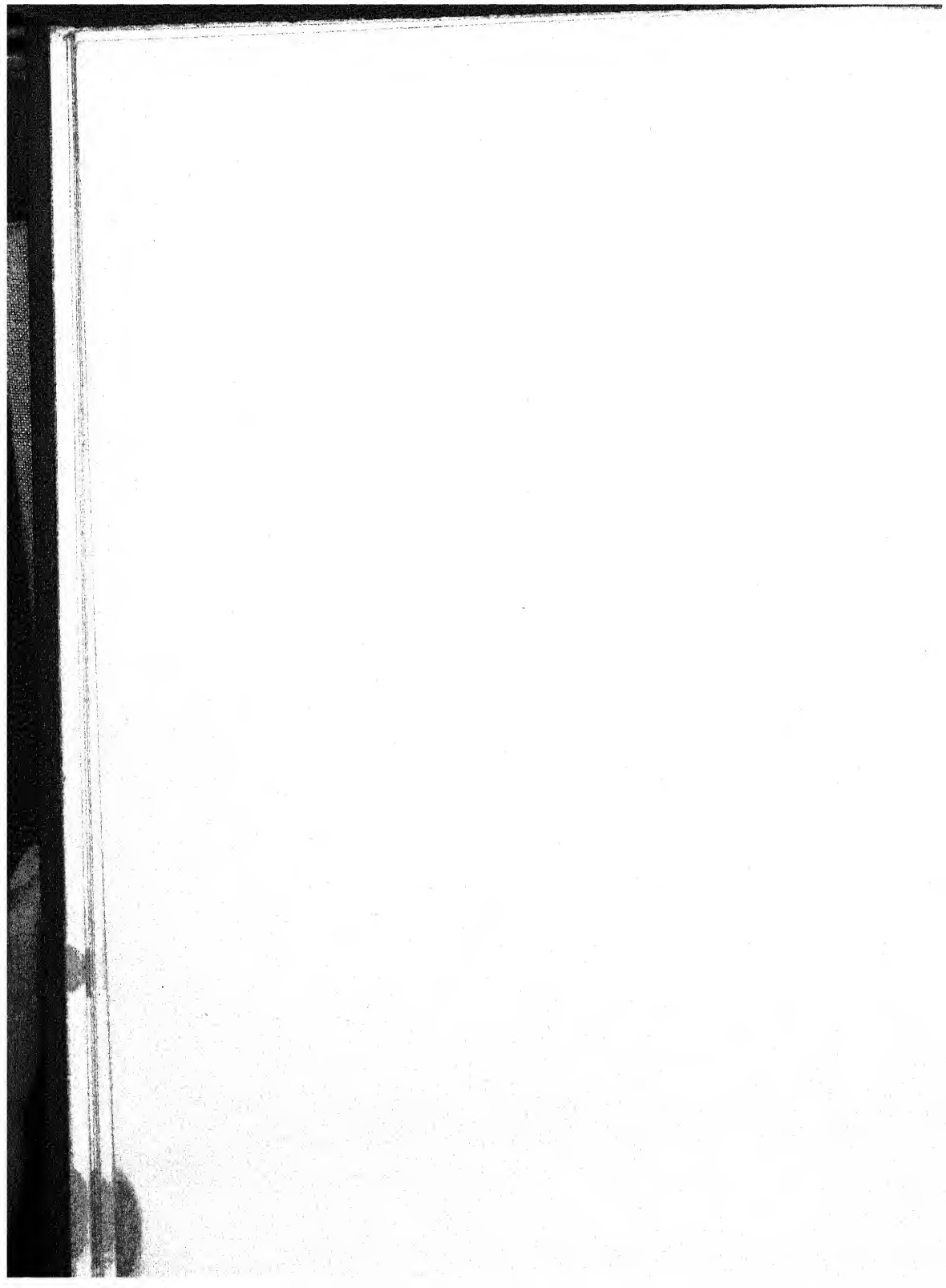
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ERRATA

- Volume I, page 691, foot of page: the upper NH_2 groups in the left and center formulae should be changed to NH .
- Volume IV, page 279, line 12: *for ester, read ether.*
Page 489, line 8: *for benzoate, read bezoar.*
- Volume V, page 44, line 3: *for Also, read Aldo.*
Page 50, line 14: *for pomatio, read pomatia.*
Page 123, line 16: *for trioxalatocobaltiate, read trioxalatochromiate.*
Page 123, line 22: *for σ , read o.*
Page 128, line 7: *for gluten, read gluten.*
Page 362, second line from bottom: *for VI, read VII.*
Page 454, line 1: *for carbonylic, read carboxylic.*
Page 455, line 8 from bottom: *for aminio, read amino.*
Page 456, line 20: *for karnirin, read kanirin.*
Page 470, line 19: *for molecules, of serine, read molecules of serine.*
Page 471, line 11: *for amino, read diamino.*
Page 473, line 17 from bottom: *for same, read name.*
Page 485, line 7: *for water, read alcohol.*
Page 504, line 2: *for galicide, read galiside.*
Page 570, line 13: *for 8.65, read 8 to 65.*

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PERMEABILITY*

BY RUNAR COLLANDER

University of Helsingfors, Helsingfors, Finland

This review, covering the greater part of the years 1935 and 1936, deals mainly with the protoplasmic permeability of plant and animal cells. The behaviour of the skin, intestine, kidney, and other complicated animal organs will not be considered. Regrettably, many references must be omitted for lack of space.

Methods of investigation.—First of all, some new methods of determining permeability deserve to be mentioned. Lucké, Larrabee & Hartline (1) have elaborated an ingenious diffraction method for studying osmotic volume changes of living cells. The method consists in illuminating a suspension of cells, e.g., sea-urchin eggs, by a parallel beam of monochromatic light and measuring the angular dimensions of the resulting diffraction pattern from which the average volume of the cells may be computed. The method is far less laborious and possesses several advantages over direct measurement of individual cells. The observations can be made at intervals of a few seconds. Another optical method for studying volume changes of uniform cells, in this case of erythrocytes, was devised by Ørskov (2). His method is based on the fact that the transmission of light through a suspension of erythrocytes decreases when the cells shrink. By measuring the transparency of the suspension continuously with a selenium photo-cell it is possible to follow very rapid volume changes of the erythrocytes.

The accurate mathematical treatment of questions of permeability, and of diffusion processes in general, often presents great difficulties to biologists. It will, therefore, be greatly appreciated that so prominent an investigator in this field as Jacobs (3) has recently taken the trouble to prepare an extensive account of the mathematical treatment of diffusion processes "with the peculiar need and . . . the limited acquaintance with the higher mathematics of the average biologist in mind." Also two papers of Resühr (4, 5) gives some useful hints concerning the calculation of permeability constants on the basis of osmotic volume changes of the protoplasts.

Permeability to non-electrolytes.—It is but natural that the first

* Received January 13, 1937.

investigators in the field of cell permeability generally confined themselves to a more or less detailed study of a single object which seemed, to them, especially well suited for experiments of this kind. Later on a comparative study of the permeability characteristics of different objects was started. With plant objects this study was inaugurated by Höfler. During the period to be reviewed here, two important investigations of this kind have been published by the botanists Hofmeister (6) and Marklund (7). These investigations show a close resemblance to each other and, therefore, they will be discussed together. In both cases the plasmolytic method was used, Hofmeister using the plasmometric, and Marklund the "incipient plasmolysis" modification of it. Both authors made a thorough study of nine different objects. Hofmeister worked mainly with elongated parenchyma cells from the stem of higher plants, whereas Marklund included among his objects, also, one moss species and representatives of several algal classes. In both cases, about a dozen non-electrolytes, representing different lipid solubility and different molecular size, were used. In regard to the main results, the agreement between the two authors is perfect. In all cases the lipid solubility (or some factor varying in close accordance with it) is the factor which determined chiefly the penetration power of the substances studied, but also the molecular size had a marked effect in that the smallest molecules permeated distinctly more rapidly than greater molecules of the same lipid solubility.

In spite of this general conformity between all the different kinds of cells examined, there were also unmistakable differences. These were, however, only differences of degree and they can, at least for the most part, be easily explained on the basis of the lipid-sieve theory. When, for example, urea in some few cases permeates faster than methyl urea this seems to indicate that the plasma membrane of these cells contains a considerable number of pores which are permeable to the molecules of urea but not to those of methyl urea. Again, when in the case of *Melosira* (a diatom), substances like erythritol and sucrose, which have an extremely low lipid solubility and a considerable molecular volume, permeate unusually fast (though also in this case distinctly slower than the more lipid-soluble and smaller molecules) this fact points to the occurrence in *Melosira* of plasma-membrane pores of an extreme width. Also the fact that some kinds of cells are found to be especially permeable to all amides tested can be easily explained on the assumption that the plasma-membrane lipoids of

these "amidophilic" cells are especially acid in character, as it is known that the addition of an organic acid to a neutral oil increases markedly the solubility of amides in the oil.

Other plant cells were studied with regard to their permeability to a variety of non-electrolytes by Wahry (8) and Resühr (9). The results of these investigators are likewise compatible with the lipid-sieve theory.

In a plasmometric comparison of the permeability of numerous different sorts of plant protoplasts to water and monovalent alcohols (especially methyl and ethyl alcohol), Zehetner (10) found that two quite opposite types of behaviour can be distinguished in this respect. Some protoplasts, when transferred from the alcohol-free plasmolysing solution into a similar solution containing alcohol, first swell suddenly and then shrink gradually to their initial volume. Other kinds of protoplasts behave in just the opposite manner: the alcohol first causes them to contract, then a slower recovery follows. Protoplasts of the first type, the "expansion type" of Zehetner, seem to be much more permeable to alcohol than to water while, on the contrary, protoplasts of the second type ("contraction type") seem to be distinctly more permeable to water than to alcohol. Curiously enough, protoplasts of the contraction type were often found to change during the experiments into the opposite type, this change probably being due to a rather sudden increase in the permeability of the protoplasts to alcohol. The opposite change, viz., from expansion to contraction type, was never observed.

The permeability of plant protoplasts to water is generally calculated from the rates of their plasmolysis and deplasmolysis. To overcome the difficulties associated with such experiments Levitt, Scarth & Gibbs (11) isolated the protoplast from its cell so that it formed a freely floating perfect sphere. With this technique the area and the volume of the protoplast could be easily calculated and there was nothing to prevent free diffusion of the liquid. In order to obtain rapid and accurate measurements a motion-picture camera was used to take photomicrographs at precisely known time intervals. The protoplasts of the large pulp cells of onion scales were used as experimental objects. Their permeability to water was found to be 21μ per hour under a pressure difference of 1 atm. at about 20°C . This value is probably the most accurate one so far obtained with plant cells. It agrees well with the results of earlier workers.

The isolation of heavy water (D_2O) in relatively pure form

immediately suggested a study of the relative permeability of living cells to D_2O as compared with H_2O . Lucké & Harvey (12) measured carefully the rate of volume increase of unfertilized sea-urchin eggs in artificial hypotonic sea water and in hypotonic sea water of the same strength but made up with 99.5 per cent D_2O instead of H_2O . They found that the rate of penetration of D_2O is the same as that of H_2O , the possible difference being less than 1 per cent. On the other hand, Parpart (13) and Brooks (14), working with mammalian erythrocytes found that the rate of hemolysis is considerably lower when the erythrocyte suspension is diluted with D_2O than when H_2O is used. Using a carefully elaborated technique Parpart thus found that D_2O penetrates the erythrocytes of cattle and rats about 44 per cent more slowly than H_2O . He suggests that this difference may be attributed partly to the viscosity of D_2O , which is approximately 31 per cent higher, and partly to the mobility of D_2O molecules, which is supposed to be 11 per cent lower, than that of H_2O . Whatever the cause might be the discrepancy between the results of Lucké & Harvey and those of Parpart and Brooks, remains to be cleared up.

Permeability to electrolytes.—The permeability of living cells to electrolytes, especially to strong electrolytes, is not yet even nearly as well known as their permeability to non-electrolytes.

Osterhout and his associates continue their important work on the permeability of large plant cells. It is possible to distinguish two closely connected lines in this work. One of them bears upon the kinetics of penetration of weak acids and bases. It has been shown by Jacques (15) that the rate of entrance of hydrogen sulfide into cells of *Valonia* is initially directly proportional to the concentration of molecular hydrogen sulfide in the external solution. On the other hand, a weak base, like ammonia, behaves in a different manner. Osterhout (16) has found that the rate of entrance of this base into the cells of *Valonia* fails to increase as rapidly as the concentration gradient of molecular ammonia plus ammonium hydroxide, although it should do so if the mode of entrance merely involved the diffusion of these substances through a non-aqueous layer. Osterhout therefore assumes that while weak acids like hydrogen sulfide enter as such, ammonia, on the other hand, combines with an acidic constituent HX of the protoplasm to form NH_4X which diffuses inward. Thus, according to this hypothesis, the rate of entrance of ammonia depends on the concentration of NH_4X in the non-aqueous layer rather than on that of ammonia in the external solution.

Another part of the work of Osterhout and his associates concerns the permeation of strong electrolytes. It was shown by Jacques (17) that a strong base like guanidine is also taken up by the cells of *Valonia* relatively faster from dilute than from concentrated solutions. This is explained, just as in the case of ammonia, by the assumption that the entrance is preceded by a reversible action between the penetrating base and one or more acidic constituents of the protoplasm. Osterhout is inclined to extend this explanation even to inorganic bases like, for example, potassium hydroxide. He had shown in previous papers (cf. 18) that an increase of the pH of the surrounding sea water increases the rate of penetration of potassium into the *Valonia* cells. He therefore suggested that the entrance of potassium may depend on the activity gradient $[K_o][OH_o] - [K_i][OH_i]$ where the subscripts *o* and *i* indicate outside and inside, respectively, potassium entering the cells as potassium hydroxide. From this standpoint, however, it is rather surprising that in the case of the freshwater alga *Nitella*, the rate of entrance of potassium is generally independent of the external pH between pH 6 and 8 as found by Jacques & Osterhout (19). They also found that when the external concentration of potassium is as low as $1 \times 10^{-4} M$ and the external hydroxyl activity is 1×10^{-8} , potassium still enters, although the internal concentration of potassium is about $10^{-1.3}$ and the internal hydroxyl activity is not less than 10^{-9} , so that the product $[K][OH]$ is higher inside than outside. It thus seems rather difficult to assume that potassium enters the *Nitella* cells chiefly as potassium hydroxide, such an assumption being rendered possible only if we suppose that for some unknown reason the "effective" internal product $[K][OH]$ is less than the product calculated from the hydrogen ion activity and the potassium concentration. Furthermore, it is pointed out by the authors that in fact all the gradients between the sap and the external solution are unfavourable to the entrance of potassium with the single exception of the potassium bicarbonate gradient. However, on other grounds, the entrance of potassium bicarbonate is not considered probable. Later on it was found by Jacques (20) that when the cells of *Nitella* are exposed to very dilute solutions of potassium (only 0.00001 N) an increase of the outer pH from 6 to 8 even diminishes the tendency of potassium to enter the sap. There is, thus, a discrepancy between the results arrived at with *Valonia* and with *Nitella*. This discrepancy has not yet been satisfactorily explained. Osterhout's views are summarised in a recent paper (21).

The behaviour of *Valonia* towards strong electrolytes has been studied also by some other authors. Thus Ullrich (22) studied the penetration of nitrate, bromide, and salicylate anions. He mentions the rather surprising observation that when *Valonia* cells are placed in sea water containing nitrate the nitrate concentration of the sap reaches exactly that of the outer solution in five days and then remains at this level. Bromide is found to penetrate even faster and the salicylate anion somewhat more slowly. It is also claimed that the intake follows the simple diffusion equation of Fick, the amount of a given anion entering the cell in each time element being proportional to the difference $c_o - c_i$ where c_o denotes the concentration of the anion in the outer solution and c_i its concentration in the sap. It may, however, be advisable to await further confirmation of these findings before they are definitely accepted.

Brooks (23) also reports some rather unexpected observations on *Valonia*. When the cells were placed in sea water plus small amounts of rubidium chloride their protoplasm during the first two days took up rubidium at a considerable rate, while its passage into the vacuole was only about one-tenth as fast. Rubidium was thus accumulated in the protoplasm to a very striking extent. In fact, the author assumes that the accumulation ratio for the protoplasm presumably reached a value of several hundred so that the solubility limit of rubidium as chloride must have been approached. After the second day rubidium was lost from the protoplasm both to the sap and especially to the external solution. Further experiments are planned by the author to confirm these interesting but still somewhat provisional results.

Several recent papers deal with the permeability of the erythrocytes to ions. The prevalent view that the cation permeability of these cells is negligible as long as they are entirely uninjured, has sometimes been questioned. It seems, however, that the recent investigations of Davson (24), Maizels (25), and Streef (26), on the whole, confirm the classical conception of the impermeability of the red cells to cations under normal conditions. The young erythrocytes, however, possibly form an exception in this respect. At least Henriques & Ørskov (27) conclude from their experiments that the erythrocytes, during the first days after their entrance into the blood, are permeable to potassium.

To account for the selective permeability of the erythrocytes to anions it has been generally assumed that the plasma membrane of

these cells may possess some sort of pores just as is the case with collodion membranes which are selectively permeable to cations. It could, therefore, be assumed that the different permeation rates of the different anions through the red cell membrane would depend on their different size, exactly as Michaelis showed that the permeability of the dried collodion membrane to inorganic cations depends on their size. To prove this assumption Höber (28) studied extensively the collodion membrane as well as the erythrocytes in regard to their permeability to organic anions. The collodion membranes used had been rendered selectively permeable to anions by adding a basic dye-stuff. The permeability of the red cells was estimated by measuring the rate of hemolysis in ammonium salt solutions and the permeability of the collodion membranes by following the membrane potentials. It was found that the penetration through both kinds of membranes was slow in the case of anions of strong carboxylic and hydroxy carboxylic acids like citrate or lactate. On the other hand, the anions of the fatty acids and of the aromatic carboxylic acids penetrate rapidly. Also the anions of the strong sulfonic acids like benzene-sulfonate or naphthalenesulfonate pass much more rapidly than could be expected from their speeds of migration. From these results Höber concludes that the ionic volume can not be regarded as the main factor controlling penetration through the collodion membrane or through the plasma membrane of the erythrocytes. Apparently, surface activity and hydrotropic character of the ions influence greatly their penetration power. The permeability of the erythrocytes, though in many respects consistent with the permeability of the selectively anion-permeable collodion membrane, differs from it in that the entrance of the ammonium salts of the more lipid-soluble fatty acids and aromatic carboxylic acids is especially favoured. This can be explained by assuming that whereas the anions pass through the pores of the plasma membrane the lipid-soluble molecules of the free acids pass through the lipid elements of the surface mosaic. As the influence of the lipids is more evident with the erythrocytes of sheep and ox than with those of man, rat, and mouse it seems natural to assume that in the former case a relatively greater area of the cell surface is occupied by lipids than in the latter.

Permeability changes.—The literature is overflowing with statements concerning more or less significant changes in the permeability of living cells. However, as pointed out by Huber (29), there are in reality few incontestably established cases of such changes and es-

pecially few that can be reproduced at will. We will consider here permeability changes brought about by (a) internal causes, (b) temperature changes, (c) light, (d) chemical agents, and (e) miscellaneous factors.

a). Marklund (7) observed some striking permeability changes occurring spontaneously during the normal course of development of the leaf cells of *Elodea* and *Taraxacum*. Thus the permeability to glycerol increases nine times and that to urea forty-six times during the early development of the *Elodea* cells; in older leaves the permeability again decreases. It seems that these permeability changes are due not only to changes in the pore diameter but also to variations in the acidity of the plasma-membrane lipoids. It still remains to be ascertained whether such spontaneous permeability changes characterize only a few sorts of cells or whether we are confronted here with a phenomenon of general occurrence.

b). The temperature coefficient of cell permeability seems to vary within fairly wide limits and is often remarkably high. (Q_{10} values of about 2 to 3 have often been found.) An interesting theoretical explanation of these facts is suggested by Danielli & Davson (30). According to these authors the plasma membrane may be considered as a potential barrier through which only molecules having more than a critical energy of translation are able to pass. This critical value will be the smaller the lower the resistance encountered by the molecule in penetrating the film. Raising of the temperature must, therefore, make possible the passage of a greater fraction of the total number of molecules in a case where the critical energy is large (and the permeability is small) than in the opposite case.

Jacobs and coworkers (31) studied the temperature coefficients of hemolysis for eleven species of mammals in solutions of certain penetrating non-electrolytes. Their results are very remarkable. With respect to the behaviour of the erythrocytes in isosmotic glycerol solutions, two sharply separated groups of mammals were found. The first is characterized by rapid hemolysis, by a low temperature coefficient, and by a very strong retardation of the process by carbon dioxide and other acids, as well as by traces of copper. The second group shows slow hemolysis, high temperature coefficients, and no retardation, but frequently even an acceleration, by the substances mentioned. Retardation in the first group is accompanied by striking increases in the magnitudes of the temperature coefficients. A critical examination of the experimental facts leads the authors to the as-

sumption that these changes in the rate of hemolysis are due to true changes in the permeability of red cells. The mechanism of these permeability changes has not yet been ascertained. On the whole, the observations of Jacobs *et al.* are in good agreement with the theory of Danielli & Davson cited above.

Döring (32) studied the influence of low temperatures on the uptake of water through the roots of higher plants and found that a sudden cooling of the roots from 20° to 0–1°C. causes a slow decrease of the water uptake, the new equilibrium being established only after thirty-five to forty minutes. She concludes that the permeability of the root cells to water is only gradually decreased. The experimental results of Döring were confirmed by Rouschal (33).

A recent paper of Levitt & Scarth (34) on permeability in relation to frost resistance and the seasonal cycle of plants deserves particular attention. Using the plasmolytic method the authors compared carefully the protoplasmic permeability of cells in the unhardened and hardened state. The material studied represented various degrees of cold resistance, from tender annuals to hardy trees. The plants, besides being subjected to artificial hardening in a cold chamber, were also examined throughout the whole year in their natural state. Permeability was discovered to increase greatly with hardening. Indeed, frost resistance and permeability were found to vary together in the life of the plant, not only in the normal seasonal rhythm and in relation to temperature changes but also in response to various other factors, such as water supply, nutrition, and even disease. It seems likely, therefore, that the permeability test will prove to be of practical value in predicting hardiness. While the permeability to salts and polar non-electrolytes with small molecules, such as water or urea, is conspicuous there is no permeability change towards apolar substances, such as urethane. These relations point to a widening of the aqueous pores or increased hydration of the plasma membrane as the mechanism of permeability increases.

c). The results of Wahry (8) concerning the influence of daylight on the permeability of the leaf cells of *Hippuris* to different non-electrolytes are so irregular that one is inclined to doubt whether the "light" and "dark leaves," compared with each other, actually were strictly comparable. Brauner & Brauner (35, 36) studied the influence of artificial illumination on the permeability of the parenchyma of beet and carrot roots. They found an increase of permeability to water and a decrease of permeability to sugar. The interpretation of

their experimental results seems, however, somewhat doubtful (29, 37).

d). The influence of different cations upon the permeability of plant cells to water has recently been studied by Baptiste (38) and de Haan (39). The experiments of Baptiste were performed with discs of potato tuber and carrot. The discs were soaked over night in hypotonic solutions of chlorides of potassium, sodium, ammonium, magnesium, and calcium. The suction pressure of the discs was then raised to a known uniform level by controlled evaporation over calcium chloride, and the uptake of water during five minutes, after immersion in distilled water, was followed by successive weighings. The effect of the cations on permeability followed the series $K > NH_4 > Na > \text{Control} > Mg > Ca$. The results, on the whole, agree with the Hofmeister series. (It should, however, be noted that for some unknown reason the discs did not recover their original fresh weight even after prolonged immersion in water.) De Haan made his experiments with epidermis cells of the onion scale. The cells were first plasmolysed in a sucrose solution, whereupon the rate of deplasmolysis was measured, partly in a pure hypotonic sucrose solution and partly in sucrose solution containing varying concentrations of sodium nitrate, calcium nitrate, or cobaltic ammonium chloride $Co(NH_3)_6Cl_3$. It was found that different concentrations of the same salt affect the protoplasmic permeability very differently. Thus the bivalent and trivalent cations decrease the permeability in low concentrations but increase it in higher concentrations. With sodium nitrate, on the other hand, only accelerating effects were observed. The author points out that the results agree well with the hypothesis that the plasma membrane consists of a phosphatide auto-complex coacervate since the points of minimum permeability coincide with the points of reversal of charge of the phosphatide system.

Stewart & Jacobs (40) confirm the observation of McCutcheon & Lucké that the permeability of the sea-urchin egg to water is greater in solutions containing potassium chloride or sodium chloride alone than in those containing calcium chloride or sea water. They found, however, that changes in the electrolyte content of the medium have very little effect on the permeability of the cell to ethylene glycol. On the other hand, after fertilization, the permeability of the eggs to water and glycol is increased almost equally. It seems reasonable to suppose, therefore, that the effect of electrolytes on the plasma membrane is of a different nature from that of fertilization. The influence of

electrolytes on the permeability of sea-urchin eggs to water was studied also by Fukuda (41).

Mond claimed in 1927 to have observed that at alkaline reactions the selective anion permeability of the erythrocytes is changed into a selective cation permeability. Recently Davson & Danielli (42) pointed out that the experiments of Mond are unsatisfactory and his conclusions unwarranted. Using an improved technique Davson & Danielli showed experimentally that erythrocytes, washed with buffer solutions at pH 8.3 to 10.1, actually lose no more potassium than they do when washed at physiological pH. Hence, the alleged cation permeability of erythrocytes in alkaline solutions is nonexistent.

A striking increase of the permeability of the erythrocytes to potassium and rubidium, brought about by carbonic acid in the presence of minute traces of lead salts, is reported by Ørskov (43).

e). It is often assumed that the rate of water filtration through a membrane is directly proportional to the suction potential applied, the resistance of the membrane remaining unaltered by the suction pressure used. Brauner (44) points out that this assumption does not necessarily hold good, as high suction pressures may reduce the hydration of the membrane and thus increase the resistance. Experiments with the testa of *Aesculus* and with the living parenchyma of beet roots were found to confirm these considerations. The results obtained with the latter object were especially striking. The exosmosis of water was in this case determined by means of a mirror arrangement which enabled the exact measurement of the contraction of the tissue cylinder in sugar solutions of different concentrations. It was thus found that an increase of the sugar concentration from one to two mols not only does not increase, but even slightly decreases, the rate of water exosmosis. These results do not agree, however, with the exact determinations of the permeability of onion-scale protoplasts to water carried out by Levitt, Scarth & Gibbs (11). It is true that, when determined at different stages of deplasmolysis (or deplasmorrhysis), the rate of penetration of water showed a gradual rise to a little beyond the normal size of the cell, and then a sharp jump upwards. However, at different stages of plasmorrhysis (contraction) the permeability remains almost constant. The authors conclude that the rise in permeability with the degree of deplasmorrhysis is primarily a mechanical effect of the extension of the membrane, which increases with both degree and rate of stretching. Increased hydration is unimportant, or else its effect would show in plasmorrhysis.

In view of the fact that plasmolytic methods are very much used in determining the permeability of plant cells it is important to note that Schmidt (45) has made a careful study of the much discussed question of whether the permeability of plant protoplasts is changed by plasmolysis. He found that the permeability of moderately-permeable cells remains almost unchanged by plasmolysis, but that the permeability of highly-permeable cells is considerably decreased, especially when a strong plasmolysis of long duration is used. Comparing different plasmolytica he found that the permeability-lowering effect increases in the following series: $KCl < NaCl < LiCl < \text{glucose} < SrCl_2 < BaCl_2 < CaCl_2$.

Model studies.—Some studies on artificial membranes deserve to be mentioned here as they may lead to a better understanding of the permeability properties of living cells. A splendidly written survey of the work performed in this field previous to 1936 is given by Höber (46).

Wilbrandt (47), working in the laboratory of Michaelis, has studied the permeability of the dry collodion membrane to ions. He explains its selective permeability to cations on the ground of a quasi-crystalline structure of collodion, the ONO_2 groups acting as dipoles with the negative charge directed towards the intermolecular spaces, no matter whether these pores are of molecular dimensions or larger. An adsorption of anions, as involved in the original theory of Michaelis, is thus no longer postulated. From solutions of collodion, mixed with basic dyestuffs or alkaloids, membranes can be obtained which, in contrast to the pure collodion membranes, are more permeable to anions than to cations. Very interesting is the observation that membranes can be built which, exactly as the living protoplasm, give a high potential difference even between two identical electrolyte solutions. The asymmetry here lies within the membrane and is artificially produced by gluing together an ordinary collodion membrane and a membrane previously impregnated with a basic dyestuff or an alkaloid.

It is generally assumed that the selective permeability of a membrane is due either to its action as a sieve or to its action as a solvent. Wilbrandt however emphasizes that there is no sharp contrast between these two mechanisms. He regards—going perhaps a little too far in this respect—the sieve theory and the solubility theory solely as two aspects of the same thing. He seems to be right, however, in pointing out that there are intermediate links between the two processes. At interfaces strong molecular forces are active, resulting in distinct

orientations of the molecules. The space properties of the molecules, which cancel out in a homogenous system due to random distribution, here add up and become an outstanding property of the system. This not only holds for monomolecular films but for all interfaces where polar molecules are involved, thus probably for the plasma membrane also. For this reason it seems to the author to be preferable not to use the concept of a homogeneous solution for the interpretation of cell permeability, but to regard the intermolecular spaces as pores and to derive the conditions in these pores from the properties and force fields of the adjacent molecular groups.

While the theory of Wilbrandt is mainly qualitative in character, Meyer & Sievers (48) present a quantitative theory of the permeability of membranes to ions. They treat the permeability of a membrane, which consists of a framework of molecules carrying laterally-fixed ionised groups, as a function of (*a*) the concentration of the fixed ions, (*b*) the width of the pores, (*c*) the outer electrolyte concentration, and (*d*) the solubility of the ions in the membrane. They report potentiometric permeability measurements which are in good agreement with the theory advanced. Teorell (49) arrives at similar conclusions in regarding the penetration of ions across a membrane as a case of diffusion in an electrolyte mixture. He expresses, as a first approximation, the membrane effect as that of an added electrolyte affecting the diffusion of other electrolytes (those under investigation). In a later paper Meyer, Hauptmann & Sievers (50) describe theoretical and experimental investigations on the permeability of liquid non-aqueous layers to ions. They deny the existence of liquid layers that are selectively permeable to cations or anions and state that the permeability of an oil layer to an ion depends on the mobility of this ion in the oil and on its partition coefficient, oil/water.

The phosphatide double films studied by Bungenberg de Jong & Bonner (51) arouse much interest also from a biological point of view. Such a double film can be considered as a bimolecular coacervate layer of phosphatide molecules which are oriented in such a manner that their hydrocarbon chains are directed outwards, thus forming on each side of the film an outer zone which is pronouncedly lipophilic in character and may contain, between the phosphatide-hydrocarbon chains, "sensitizer molecules" as, for example, cholesterol or triolein. The ionised groups of the phosphatide molecules, on the other hand, are directed inwards and thus constitute a central

zone of hydrophilic character. Bungenberg de Jong suggests, as a working hypothesis, that the special properties of the protoplasmic membrane depend upon one or more such phosphatide double films. This hypothesis would in fact explain the well known immiscibility of the aqueous protoplasm with the surrounding aqueous medium. It is also pointed out that there are several analogies in regard to the effect of electrolytes and non-electrolytes upon the solvate content of the coacervate on one hand and upon cell permeability on the other. Theoretically it seems also probable that the permeability of the phosphatide coacervate films may be, in a general manner at least, similar to that of the living protoplasts. Unfortunately, however, the permeability of the artificial phosphatide double films has not been studied experimentally, so at present it is not possible to decide definitely how close the resemblance really is between the permeability properties of these films and those of living cells.

It is also regrettable that as yet so very little is known about the permeability of other kinds of artificial mono- and bimolecular lipid films. A suitable method for obtaining thin lipid films expanded between two aqueous phases has been found recently by Danielli (52) and it is hoped that this gap in our knowledge soon will be filled.

The composition and structure of the plasma membrane.—It is now almost unanimously assumed that some sort of lipoids form an essential part of the plasma membrane. Frey-Wyssling (53) and Ullrich (54) prefer, however, the more vague expression "hydrophobic substances" which includes, besides all sorts of lipoids, also, for example, hydrophobic proteins.

Measurements of the electrical impedance of suspensions of various kinds of cells, such as erythrocytes, leucocytes (55), sea-urchin and starfish eggs (56, 57), yeast cells (58), etc., make it probable that the poorly-conducting surface membrane is only a few molecules thick. The extensive work of Osterhout and his associates (59) on the electrical phenomena in large plant cells should also be mentioned in this connection.

An interesting discussion of the properties of lipid films in relation to the structure of the plasma membrane is given by Danielli (52). In his opinion the considerable changes in surface area of the cells, for example in osmotic swelling, show that the plasma membrane must be more than one molecule thick (probably more than two); on the other hand the sudden incidence of leakiness at a certain point in osmotic swelling clearly suggests that the film is not more than three

or four molecules in thickness. Furthermore, he points out that both continuous lipid films and mosaic films are metastable, and that the former type of film is more stable than the latter, but that it is impossible to exclude the mosaic structure of the plasma membrane on the basis of mechanical properties only. On the other hand, he calls attention to the weakness of one of the chief reasons for considering the plasma membrane to be a mosaic, viz., the fact that it permits the passage also of some "lipoid-insoluble" substances. In fact, continuous lipid membranes also, when thin enough, have been shown to be permeable at least to water.

Curtis (60) found that when erythrocytes swell in hypotonic solutions so that their area increases, the capacity per unit area is nevertheless almost unchanged. It seems, therefore, that the semi-permeable plasma membrane does not undergo thinning when it is stretched, but that it is augmented during its extension by a transfer of materials from neighbouring regions of the cell.

Permeability and active transport of matter.—The word permeability as used in the foregoing pages involves an inert membrane, freely permeable to some substances, less or not at all to others. It is felt, however, that this review would not be complete if it were not explicitly pointed out that the living protoplasts actually very often play a much more active rôle, forcing water or solutes to move in a certain direction, even against the concentration gradient. There can be little doubt as to the eminent importance of such processes and it may therefore be appropriate to mention here, very briefly, some recent investigations which have substantially contributed to a proper understanding of the active transport of matter as contrasted to the main cell permeability. Especially in the important investigations of Hoagland & Broyer (61), Steward, Berry & Broyer (62), Prevot & Steward (63), and of Rosenfels (64), the dependence of salt accumulation upon aerobic respiration is clearly shown. Apparently, however, the carbon dioxide output *per se* is not the decisive factor. On the contrary, the energy value of the oxidation is the factor most probably involved, not merely because it supplies the energy expended in the accumulation alone, but rather because it maintains the vital activity and growth with which salt accumulation in plant cells seems to be inevitably associated.

In this connection some interesting observations of Arens (65) may also be mentioned. He found that the lower sides of photosynthesizing leaves of water plants, e.g., *Potamogeton*, absorb all the

different constituents of a calcium bicarbonate solution, viz., Ca^{++} , HCO_3^- , $\text{CO}_3^{=}$, and CO_2 , in about the same proportions as they occur in the surrounding solution. At the same time calcium hydroxide is excreted from the upper side of the leaves into the solution. There seems thus to be a distinct polar transport of matter through the leaves. The importance of these unexpected findings makes a further confirmation desirable.

An interesting case of water translocation against the osmotic gradient has recently been discovered by Walter & Steiner (66). They found that the seedlings of viviparous mangrove trees are supplied with water from the mother plant although the osmotic value of the sap of the seedlings is only about one-half that of the mother plant and much lower than that of the soil solution. The details of this process of water translocation still remain to be ascertained.

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BIOLOGICAL OXIDATIONS AND REDUCTIONS*

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THE CHEMISTRY OF THE COFERMENTS (PHOSPHOPYRIDINE NUCLEOTIDES)

By the use of the methods developed by Warburg, Christian & Griese (1) to study the spectral changes due to reversible reduction of the pyridine group in coferments, a rapid advance has been made in this field. It is now definitely established that cozymase, though chemically different from Warburg's coferment, contains the same active pyridine group (2, 3).

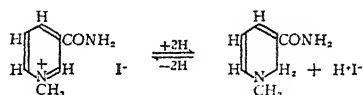
Because of their constitution, this group of catalysts is called phosphopyridine nucleotides by Warburg (4).

The pyridine part of the phosphopyridine nucleotides.—Karrer and Warburg (5) continued their search for model pyridine compounds similar to the catalytically active pyridine in coferments. Among the compounds studied, only those containing a quaternary nitrogen were found to undergo reversible oxido-reduction. The closest resemblance was found with nicotinic acid amide methiodide. Like the coferment itself it is easily reduced with sodium hydrosulphite, and reoxidised by flavoprotein (yellow enzyme). An absorption band, similar to that of the coferments, appears on reduction and disappears on oxidation. The maximum is slightly shifted toward the visible. A not quite pure preparation of the reduced compound was isolated by Karrer *et al.* (6) as an iodide-free yellowish oil. Significantly, by reduction the iodide ion is lost. In alkaline solution it reduces more strongly than hydropyridine in the coferment molecule. The potentials measured with the platinum electrode were very negative, unstable, and dependent on pH.

With the manometric method improved by Haas (7), Warburg & Christian (4) have measured the acid formation brought about by the hydrosulphite reduction of the two pyridine nucleotides and Karrer's model pyridine. Besides the two mols of acid due to formation of acid sulphite, one additional mol of acid appeared in the case of all the three pyridines. This acid formation in the process of reduction

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leads to the conclusion that the reduction is taking place at the double bond closest to the quaternary nitrogen. Therefore the process of reduction has been formulated as follows [Karrer (6), Warburg (4)] :



As may be seen from the above scheme, the process generally described as the uptake of two hydrogen atoms is actually the uptake of one hydrogen atom and one electron. The quaternary nitrogen hereby loses its charge, and the anion previously bound to it is set free together with the remaining hydrogen ion. In this manner the additional mol of acid appears.

Since the pure coferments have been found to be free of anions other than the phosphate bound in the molecule (4), the appearance of the hydrogen ion in the reduction process indicates that the nitrogen in the oxidised compound forms an inner salt with the phosphoric acid. The methyl group attached to the nitrogen in the model is supposed to be replaced in the coferments by a carbohydrate.

It is of value to point out the significance of the stability conditions of the pyridine nucleotides as found by Warburg, Christian & Gries (1) with their coferment, and likewise for cozymase by Warburg & Christian (4), Adler *et al.* (8), and Green (9). The oxidised form, supposedly containing a strongly basic nitrogen, is stable in acid solution as the ion, but very unstable in alkaline solution where it is partly undissociated. The reverse is the case with the reduced form which contains a weakly basic nitrogen and which is extremely unstable in acid (partly ionised) but stable in alkali (unionised). This might be explained in the following manner: the oxidised form cannot tolerate the addition of one electron alone, nor the reduced form the loss of one electron alone without causing greater changes in the molecule.

Adler, Hellström & Euler (8) describe the appearance of a transient yellow color on addition of sodium hydrosulphite to cozymase in slightly alkaline solution. This yellow color persists if the reduction is carried out at pH 13. The alkaline reduction product differs from cozymase reduced at neutrality, in that the absorption maximum is shifted to 360 mμ (the absorption band itself extends into the visible) and it reduces silver ion, and methylene blue. It was suggested that this alkaline reduction product might represent a half-reduced cozymase, corresponding to the semiquinones of Michaelis

(10). On neutralisation, oxidised cozymase appears in the solution. Karrer & Benz (11) described the formation of a transient yellow color with their model pyridines. This reaction was found to be characteristic for the pyridines able to undergo reversible reduction. These workers also mention the possibility of an intermediate oxidation step.

Myrbäck (12) continued experiments on the oxidation of cozymase with hypoiodide. With highly purified preparations it was observed that a somewhat lower consumption of iodine took place. The oxidation itself was accompanied by acid formation. Some model experiments with angelica acid lactone were also reported.

Vitamin B₁ is related to this group. Peters (13) showed that the vitamin is concerned with the oxidation of pyruvic acid in the cell. It contains a quaternary nitrogen in a thiazole ring [Williams (14)]. Lipmann (15) has shown that the vitamin reacts with sodium hydrosulphite. The reaction was somewhat slower than with the coferments; two atoms of hydrogen were used and nearly one mol of extra acid appeared. Hence the reduction should take place at the double bond closest to the nitrogen. Therefore it was assumed that the mechanism of reduction corresponds to that shown for the coferments. The equation might be written exactly in the same way as for the pyridylum compounds only substituting a thiazylum for the pyridylum nitrogen.

Surrounding groups.—The most pronounced difference between the two pyridine coferments is that Warburg's coferment contains three mols of phosphoric acid (1) and cozymase contains two (2). According to their composition, Warburg (4) has introduced the names of triphosphopyridine nucleotide and diphosphopyridine nucleotide.

Both yield on hydrolysis one mol of nicotinic acid amide, one of adenine, and two of carbohydrate, besides phosphoric acid (1, 4, 16). Triphosphopyridine nucleotide has been further purified by Warburg & Christian (4). The compound gives the Thomas test for muscle adenylic acid (1). A molecular weight of 782 was calculated for one mol of pyridine, estimated by total reduction with Pt-H₂. The nature of the constituent sugars has not been investigated. Diphosphopyridine nucleotide has been isolated from yeast by Euler *et al.* (2, 16) and from red blood cells by Warburg & Christian (17). To separate the two pyridine compounds, Euler & Adler (18) used the chromatographic method; the more highly phosphorylated compound was re-

tained by a column of alumina (Al_2O_3). Warburg obtained a clean separation using the different solubilities of the barium salts in dilute alcohol in which medium the compound with more phosphorus is insoluble. According to Schlenk (19) and Euler *et al.* (20) both carbohydrates in the molecule are ribose. The molecule contains muscle adenylic acid, and has a composition corresponding to the sum of the free components minus five mols of water. Warburg (4) is inclined to assume a combination with the loss of four mols of water. A molecular weight of 694 was calculated for one mol of pyridine, estimated by total hydrogenation with Pt-H_2 .

Cozymase and cophosphorylase.—Euler *et al.* (21) showed with several systems that pure cozymase cannot activate phosphate transport. No activation of glycolysis was found with pure cozymase alone. Warburg & Christian (4) showed conclusively that fermentation does not proceed in the presence of either adenosinepolyphosphate or pyridine nucleotide alone. Meyerhof & Ohlmeyer (22) reported that, in muscle extracts dialysed long enough, adenylic acid no longer activated glycolysis. In the same extracts they found glycolysis partly activated with greater amounts of a preparation containing 40 per cent cozymase, while much smaller amounts of this, with excess of adenylic acid, gave complete restitution. Meyerhof & Kiessling (23) isolated a compound obtained from maceration juice by the action of phosphopyruvic acid on impure cozymase, which they assume to be phosphorylated cozymase. Euler (21) was inclined to assume that this product is a phosphorylated breakdown-product of cozymase. He reported that, on alkali treatment, only nicotinic acid amide was split off [Schlenk & Euler (24)]; the remaining part activated phosphorylation and was supposed to be an adenylic acid combined with an additional ribosephosphoric acid. Ohlmeyer's (25) experiments show that adenylic acid does not activate oxido-reductive processes. His findings that with cozymase preparations phosphorylation can be activated might be due, according to Euler *et al.* (21), to impurities. There can be no doubt that the processes of phosphate and hydrogen transport are intimately connected with each other. In some way, a chemical contact [Borsook (26)] between the two catalysts should exist. According to Lohmann (27), the addition of phosphate to adenine-ribose-5-monophosphate, and the removal of phosphate from adenine-ribose-5-triphosphate, goes on in two steps. The intermediate is an adenine-ribose-5-diphosphate. He gave evidence for the assumption that the phosphoric acid groups are linked in a straight chain.

PYRIDINE NUCLEOTIDES AS CATALYSTS

From the study of the kinetics of the reaction between hexosemonophosphate, triphosphopyridine nucleotide, and specific yeast protein [Negelein & Haas (28)], Warburg concluded that the specific catalyst is a pyridine-nucleotide-protein compound. In no case has it been found that a cell substrate reacts directly with a pyridine nucleotide. The reactivity always first appears in the presence of a protein specific for the special process. The similarity of all these processes led Warburg (4) and other workers in this field to assume that, in all cases, a pyridine-nucleotide protein is the active catalyst.

The *Zwischenferment* has been purified to a great extent by Negelein & Gerischer (29).

Triphosphopyridine nucleotide.—In a study of the breakdown of phosphogluconic acid in yeast maceration juice, described by Warburg, Christian & Griesche (4), Lipmann (30) found that carbon dioxide formation takes place only in the presence of oxygen. Nearly one gram-atom of oxygen was consumed for each mol of carbon dioxide evolved. An oxidation of phosphogluconic acid to α -keto acid, followed by decarboxylation, was assumed. Simultaneously Warburg & Christian (31) showed that phosphogluconic acid is still further oxidised by triphosphopyridine nucleotide, several yeast proteins, and flavoprotein. One half-part of hexosemonophosphate is oxidised by these catalysts to water and carbon dioxide. Per mol of hexose, three mols of oxygen were consumed, and three mols of carbon dioxide evolved. Dickens (32) followed the suggestion of Lipmann (30) that the further oxidation of phosphohexonic acid indicates a new course of carbohydrate breakdown. He showed that damaged brain is no longer capable of oxidising glucose, but oxidises hexosephosphate, phosphogluconate, gluconate, and ketogluconate. With intact tissue, none of the named substances is oxidised. It is concluded that glucose is phosphorylated in order to be oxidised, and that the intermediates cannot penetrate into the cell. He also reported that, with a fraction of yeast proteins, triphosphopyridine nucleotide and flavoprotein phosphogluconic acid were oxidised with one gram-atom of oxygen per mol. The oxidation product was decarboxylated after addition of carboxylase. According to these findings, as yet not elaborated, triphosphopyridine nucleotide catalyses the oxidation of the α -hydroxy group in a phosphorylated polyhydroxy carbonic acid, and probably more generally of the aldehyde group of a phosphorylated polyhydroxyaldehyde.

Das (33) reported that glucose dehydrogenation is catalysed by both pyridine nucleotides.

Diphosphopyridine nucleotide; fermentation and glycolysis.—In the processes of fermentation and glycolysis the primary oxidation product of glucose is transformed into the fermentation oxidant which oxidises glucose to the primary oxidation product, and so on. In oxidising glucose, the oxidant (aldehyde or pyruvic acid) is reduced to the end product—alcohol or lactic acid. The primary or at least one of the primary oxidation products of glucose was isolated by Nilsson (34) who used fluoride; this inhibited only the transformation into the oxidant. After supplying the ready oxidant, aldehyde, to glucose and phosphate, the primary oxidation product, phosphoglyceric acid, was accumulated because it could not be transformed. The transformation of phosphoglyceric acid into the fermentation oxidant was demonstrated by Embden *et al.* (35). It is transformed by muscle into pyruvic acid plus phosphate and by yeast into acetaldehyde, carbon dioxide, and phosphate. Meyerhof, Lohmann & Kieselring (36) followed the transformation and were able to distinguish the following steps: Phospho-3-glyceric acid \rightarrow phospho-2-glyceric acid \rightarrow phosphopyruvic acid + phosphate. Because phosphate was found to be bound to an accumulated oxidation product, it was reasonable to assume that the reductant in fermentation must also contain phosphate. The reductant corresponding to phosphoglyceric acid is phosphoglyceric aldehyde. It remains to be shown whether a triosephosphate [Meyerhof (37)] or hexosemonophosphate [Warburg & Christian (4)] is the reductant in fermentation.

Warburg & Christian (4) have clarified greatly the reaction between the reductant and the oxidant of fermentation. To study the pure oxido-reduction they did not use an inhibitor like fluoride. Instead the enzymes catalysing the transformation of the primary oxidation product into the oxidant were removed by fractionation. By the acidification of yeast maceration juice to pH 4.3 two main fractions were derived. The acid-soluble fraction, called protein B, was not treated further except for dialysis to remove the activators. The acid-insoluble portion was carefully fractionated to a very concentrated active protein, called protein A, necessary for oxido-reduction [Negelein (38)]. The special function of the two fractions was not mentioned. To the fractions A and B, adenosine polyphosphate, diphosphopyridine nucleotide, magnesium, manganese, ammonium, and phosphate were added. In this solution the reaction between Robi-

son's hexosemonophosphate, the reductant, and aldehyde, the oxidant, was studied. Because no fermentation could take place after the removal of the transforming enzymes, the primary oxidation products of the polyhydroxyaldehyde reductant were fixed as acids. The acid formation was measured in bicarbonate solution, and the end products were determined. Also in this simplified system, for each mol of carbohydrate oxidised, one mol of hexosediphosphate appeared, fulfilling the Harden-Young equation. The new fact is that hexosemonophosphate with two mols of aldehyde is oxidised to nearly one mol of pyruvate and one mol of phosphoglycerate. Warburg & Christian (4) are inclined to assume that hexosemonophosphate first is oxidised in the middle of the molecule. It has been found that for this oxidation, carried out by diphosphopyridine nucleotide, manganese is needed [Negelein (cited in 4)]. The oxidation is intimately associated with phosphate transport to another molecule of hexosemonophosphate. This process is much more complicated than the oxidation of the same substance at the terminal aldehyde group by triphosphopyridine nucleotide.

Hexosediphosphate as reductant reacts ten times slower in this system. A slow reaction is brought about if pyruvate is used as oxidant instead of aldehyde. The system is a test system for each of the protein fractions and each of the carriers.

With the ultraviolet absorption method, reduction and oxidation were measured. It was shown conclusively that the hydrogen in fermentation is transported by diphosphopyridine nucleotide, and that this transport is intimately connected with phosphate transport. If hexosemonophosphate is added to a mixture of protein B and pyridine compound only partial reduction takes place. The reduction is not complete until adenosinepolyphosphate, protein A, and the other constituents of the test solution are added. The pyridine compound thus reduced by hexosephosphate is oxidised instantaneously by the addition of a small amount of aldehyde. In the presence of hexosephosphate in excess, reduction of the pyridine compound begins anew, followed by oxidation on the addition of new aldehyde. It is noteworthy that in this system with a comparatively low reaction rate (5 cmm. of acid per min.) the reduction is a slow process relative to the oxidation and obviously limits the rate. The rate in a well fermenting juice would be about thirty times as fast.

More than two years ago, from reductometric measurements in fermenting extracts, the conclusion had been drawn by Lipmann (39)

that the function of cozymase in fermentation is that of a hydrogen carrier.

Parallel with Warburg's investigations, in Euler's laboratory the reaction of diphosphopyridine nucleotide with fermentation substrates has been studied by the use of ultraviolet spectrophotometry. Euler, Adler & Hellström (40) showed that the hydroypyridine compound plus yeast protein is oxidised with great speed by aldehyde. On the other hand they found that triosephosphate reduces the pyridine in cozymase. Indirectly thereby the function of cozymase as the hydrogen carrier in fermentation has been proven.

Also alcohol, the end product of fermentation, was found to reduce the pyridine in cozymase. The rate of reaction was much slower than the rate of the reverse reaction between aldehyde and hydrocozymase. When high concentrations of alcohol were used the reaction came to a standstill before all the cozymase was reduced, which indicates that an equilibrium had been attained. A closer study showed the existence of a true equilibrium in the system: alcohol + cozymase \rightleftharpoons aldehyde + hydrocozymase. In this equilibrium the reverse reaction is greatly favored. Rising pH shifts the equilibrium to the right. The equilibrium constant at pH 6.3 was found to be 7.8×10^4 , at pH 7.7, 0.7×10^4 . It was pointed out that, according to the equilibrium conditions, the catalysis of aldehyde reduction, as in fermentation, would not be greatly influenced by the accumulation of the reaction product. The reverse would be the case for the catalysis in the opposite direction. As is well known, fermentation is found to be inhibited only by a high alcohol concentration, while the reduction of methylene blue by alcohol was found to be practically completely inhibited in the presence of aldehyde.

The rates of the reaction with the pyridine compound were found to be proportional to the concentration of specific protein. The position of the equilibrium was independent of the concentration of the protein.

In studying the nature of the reductant in fermentation, Euler *et al.* (40, 41) made it appear very likely that hexosediphosphate is only attacked after splitting into two triosephosphates. Protein fractions were prepared which were active in the oxidation of alcohol but inactive in the catalysis of triosephosphate oxidation. Fractions showing activity with triosephosphate, but not with alcohol, were not found.

Wurmser & Filitti-Wurmser (42) measured potentials in the system: isopropyl alcohol-acetone (with yeast enzyme). A value of

0.176 volt was found for E_0 . From the free energy data for the system, ethyl alcohol-aldehyde, the E_0 was calculated to be 0.20 volt (25°).

Lactic, malic, and fumaric acids.—The cozymase catalysis of lactic acid dehydrogenation has been studied comprehensively by Green & Brosteaux (9). It was shown that cozymase is reduced by lactic acid in the presence of heart protein. With cozymase alone no oxygen consumption appeared. Neither the complete system nor cozymase reduced with sodium hydrosulphite reacted with cytochrome. Methylene blue, pyocyanin, and flavin catalysed oxygen consumption. The maximum effect of the dyes was first reached at a fairly high concentration. Flavoprotein had very little effect, and furthermore did not increase the effect of smaller dye concentrations. Flavoprotein had a great effect on the rate of methylene blue reduction with cozymase (reduced by sodium hydrosulphite), but no effect on methylene blue reduction with lactic acid-cozymase-heart protein. Also with enzyme solutions treated several times with kaolin or kieselguhr, a considerable oxygen consumption was found with methylene blue alone. By reason of these experiments, the necessity of flavoprotein as the carrier between cozymase and dye is doubted in the case of lactic acid dehydrogenation. As a carrier between cozymase, protein and oxygen, a red oxidation product of adrenaline was found to be very active. If adrenaline were added to the complete system this red dye was formed aerobically and the oxygen consumption rose gradually. Anaerobically it was reduced by the system and was reoxidised by oxygen.

Pyruvic acid was identified as the product of the oxidation of lactic acid. The great inhibition of oxygen consumption observed in the presence of the oxidation product is significant. The rate of oxygen consumption declined rapidly, but could be stabilised by addition of a ketone fixative; 0.2 M NaCN had the best effect.

In a study of malic acid dehydrogenation, very similar conditions were found by Green (43). Cozymase was part of the catalytic system. The same extract from pig heart, as used in the lactic acid experiments, was used here; but the active protein was found to be different from that which was active in the case of lactic acid. The same dyes were used as carriers. Flavoprotein had little effect. Oxaloacetic acid was isolated as the oxidation product and, due to the inhibition by this compound, again a rapid fall in oxygen consumption and stabilisation with cyanide were observed.

A very interesting dismutation of fumaric acid to succinic and

oxaloacetic acid was found: In a system containing fumarase, malic and succinic dehydrogenase, fumarate was partly transformed into malate; then the malate reduced the fumarate, and was itself oxidised to oxaloacetate. The latter reaction only takes place in the presence of methylene blue.

Fumaric acid is not dehydrogenated unless transformed into malic acid [Green (43); Thunberg (44); Laki (45)]. Adler & Michaelis (46) reported that methylene blue reduction with lactic and malic dehydrogenase from heart is accelerated by flavoprotein if the enzyme solution had been treated with alumina (Al_2O_3).

Adler, Euler & Hellström (47) found, using the ultraviolet absorption method, that lactate reduces diphosphopyridine nucleotide in the presence of heart protein. Pyruvate oxidises almost instantaneously the hydropyridine compound. In this case, as with aldehyde, the reverse reaction is greatly favored. The position of the equilibrium in the system, lactic acid-pyridine compound-pyruvic acid, was found to be very similar to that found with the alcohol-aldehyde system.

In these cases a catalysis in both directions cannot be doubted. The same situation should be found with malic-oxaloacetic acid, as indicated by the great inhibition of malic acid dehydrogenation by oxaloacetic acid. Banga's finding (48) that oxaloacetic acid is reduced with great speed by various tissues points in the same direction. Considering the catalysis of the dehydrogenation of the reduced forms of these substrate systems, the equilibrium conditions clearly show that the free energy level of the catalyst system in the standard state lies far below that of the substrate systems. The fact that the dehydrogenation of a great number of cell metabolites is catalysed by a catalyst with the above-named properties seems at first sight very surprising, but due to this strained situation the catalyst operates easily in the direction of synthesis, in which the cell is mainly interested. An indication in this direction is given by the finding of Euler & Günther (49) that addition of cozymase increases carbohydrate synthesis from lactic acid.

Szent-Györgyi's theory of fumarate catalysis has been outlined in a preceding review. Recently he gave a summary of the theory evolved and the work done in his laboratory (50). Annau (51) showed that, with liver brei, fumarate in combination with pyruvate or alanine gave a remarkably high respiration, in many cases clearly exceeding a simple summation effect. Boyland & Boyland (52), with

tumour, and Greville (53) with various tissues, found a more or less pronounced increase in respiration with a medium concentration of fumarate. With damaged tissues the effect was found to be greater. Stare & Baumann (54) showed that very small amounts of fumarate gave an additional oxygen consumption with muscle preparations, exceeding significantly the oxygen equivalent of the added fumarate. Innes (55) repeated the experiments of Szent-Györgyi with muscle, and used a very accurate method to determine the amounts of fumarate and of related substances which remained after oxidation of fumarate. Fumarate was found to disappear in amounts greater than, or equivalent to, the amount of extra oxygen consumed. The method used by Szent-Györgyi (50) to estimate fumarate was criticised.

DEHYDROGENATION OF PYRUVIC ACID

The investigations of Peters and his colleagues (13) have brought to the front the problem of pyruvic acid dehydrogenation in animal tissues. In their important work they showed that vitamin B₁ is concerned with the oxidative breakdown of pyruvic acid. Pyruvic acid oxidation was found to be greatly improved by addition of the vitamin to avitaminotic tissue *in vitro*. With normal brain, addition of the vitamin was largely without influence; sometimes it inhibited slightly. Sherman & Elvehjem (56) confirmed the experiments of Peters, showing that pyruvic acid oxidation by avitaminotic tissue is enhanced by the addition of the vitamin.

The anaërobic breakdown of pyruvic acid in tissues was described by Krebs (57). He showed that two mols of pyruvic acid react with each other to form, in a dismutative reaction, acetic acid plus carbon dioxide on the one hand, and lactic acid on the other. Besides this dismutation, a mixed dismutation between different keto acids was considered. As another product of the anaërobic breakdown of the pyruvic acid, succinic acid was found. Succinic acid formation was tentatively assumed to be due to the intermediate formation of α -keto glutaric acid ($\text{HOOC} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{COOH}$). Among the products of the anaërobic breakdown of pyruvic acid, α -keto glutaric acid was found by Weil-Malherbe (58). He demonstrated the oxidative decarboxylation of α -keto glutaric acid to succinic acid and carbon dioxide. With avitaminotic brain Lipmann (59) showed that pyruvic acid was formed from glucose and fructose in the absence, but not in the presence, of vitamin B₁. The dehydrogenation of pyruvic acid by methylene blue with suspensions of brain cells was studied. The

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stem catalysing this reaction was found to be unstable. The rate of hydrogenation was constant down to concentrations almost equivalent with the concentration of the dye. Manometric measurements under anaërobic conditions showed that, with the disappearance of 10 mols of pyruvic acid, one mol each of an acid, carbon dioxide, and lactic acid were formed. In the presence of methylene blue no dismutation, but oxidation through dye reduction, took place. In anaërobic experiments with avitaminotic brain, no definite effect of the vitamin could be shown. No decarboxylation without oxidation or dismutation has been found with animal tissues.

Barron (60) studied the dehydrogenation of pyruvic acid with *C. freundii*. The oxygen consumption was measured. Pyruvic acid was oxidised to acetic acid and carbon dioxide. The enzyme catalysing the dehydrogenation was very unstable. Sodium fluoride and pyrophosphate inhibited strongly while potassium cyanide and carbon monoxide inhibited less than in the case of lactic acid oxidation. Hydrogen sulphide, in concentrations completely preventing lactic acid oxidation, had no effect. Quinone, indophenols, and other dyes inhibited almost completely in very low concentrations.

Cook & Harrison (61) isolated acetic acid from liver.

COLOURED HYDROGEN CARRIERS

Theorell (62) has studied the reaction between a hydroxyphenylpyridine nucleotide and cytochrome-*c*. Spectroscopically, he measured the reduction of cytochrome using hexosemonophosphate, *Zwischenferment*, and triphosphopyridine nucleotide as reductant. No reduction of cytochrome took place. The same result was obtained by Green & Brosteaux (9) under similar conditions. Theorell found that the reaction is catalysed by flavoprotein, in the presence of which cytochrome is easily reduced by the Warburg-Christian system. The rate of reduction of cytochrome was measured at different oxygen pressures. At atmospheric pressure the rates of reaction between leucoflavoprotein and oxygen or cytochrome respectively were equal. At 0.05 atm. of oxygen, cytochrome reacted four times as fast as oxygen. The oxygen pressure within the cell was calculated to be about 0.03 to 0.04 atm. At such low pressures flavoprotein reacts directly with oxygen very slowly. But the experiments showed that with cytochrome and *Atmungsferment* it might react faster. Thus, in the cell, flavoprotein could be a link between the coferment and cytochrome.

Kuhn & Rudy (63), in a study of the catalytic activity of flavins

in the Warburg-Christian system, found that unphosphorylated flavins also show activity in the presence, but not in the absence, of the protein part of flavoprotein. 150 μg . of free lactoflavin plus protein gave two-thirds of the effect given by 2.5 μg . of flavinphosphate in the presence of the same amount of protein. By studying a great number of synthetic flavins, they found that the following properties of the molecule are essential for the catalytic activity of flavins (the natural flavin is 6,7-dimethyl-9-*d*-ribitylflavin): (a) the structure of the pentose; (b) the acidic hydrogen in the -NH group in position 3; (c) the presence of at least one of the 6,7-methyl groups; (d) esterification with phosphate not essential, but in all cases the affinity to the protein was found to be greater with the phosphorylated products. Only the flavins showing catalytic activity were found to be as active as the vitamin.

According to Kuhn & Boulanger (64) the normal potential is raised enormously if flavinphosphate is bound to the specific protein. The measured values were $E_0 = 0.373$ volt for flavoprotein, and 0.187 volt for flavinphosphate. The phosphorylation of flavin was entirely without influence on the potential. Michaelis, Schubert & Smythe (65) discovered an intermediate oxidation product of lactoflavin, stable in the physiological pH range. By very careful potentiometric titrations they obtained curves from which the maximum concentration of the semiquinone was calculated to be 10 per cent at neutrality. At pH values lower than 2, the colour of the intermediate had been found to be red. At pH values higher than 2, the intermediate was green coloured. This green colour could be observed in highly concentrated solutions on reduction. It was considered probable that the semiquinone is the reactive form of lactoflavin.

Theorell (66) improved his method for the isolation of cytochrome-*c*. The preparation obtained was assumed to be a pure product. The molecular weight, estimated by the diffusion method, was found to be 16,500. Colorimetric and gravimetric measurements gave identical results. The iron content of 0.34 per cent corresponded with one atom of iron per molecule. In strongly acid and alkaline solutions cytochrome-*c* was oxidised by air. The rate of auto-oxidation increased rapidly between pH 3 and 2, and a significant change of colour took place in this pH interval. A change in the reduced product was shown by a change of the spectrum. The oxidised product changed visibly from red to brown. The auto-oxidisable acid cytochrome was revertible completely to the neutral form. Millikan (67)

howed by direct photoelectric measurements that during a tetanic contraction of warm-blooded muscle, 50 per cent of the myoglobin remained reduced. The circulation was unimpaired during the experiment. According to Theorell (62) the above-measured ratio, myoglobin: O₂-myoglobin, corresponds to an oxygen tension of 0.03 to 0.04 atm. inside the working muscle cell.

DEHYDROGENATION OF VARIOUS SUBSTRATES

Green (68) has studied the dehydrogenation of α -glycerophosphoric acid. As enzyme he used a very fine suspension of exhaustively washed and crushed muscle. No accelerating effect was observed by addition of cozymase. He assumes that this dehydrogenation is not catalysed by a pyridine compound, but is aware of the possibility that a coferment could be strongly bound in the preparation. On the other hand, cytochrome-*c* increased the oxygen consumption in proportion to its concentration (between 0.4 and 1.5×10^{-5} M); the preparation contained indophenoloxidase in great excess. This ready reaction with cytochrome and the observed non-effect with flavoprotein is taken as an argument against the action of a pyridine coferment. Also methylene blue, if used in high concentrations, catalysed the oxidation. With the dye near its maximum concentration, potassium cyanide increased the oxygen uptake slightly; with cytochrome, 2×10^{-3} M KCN inhibited completely. Much care was taken to identify the oxidation product, and, though isolation was not possible, in various ways it could be identified as a triosephosphate. The identification was only possible if potassium cyanide was used as fixative, otherwise the compound was decomposed non-enzymatically into methylglyoxal and phosphate. The difference between the enzyme from muscle and that from yeast was studied and it was found that the yeast enzyme is soluble and does not link with cytochrome. According to Wagner-Jauregg & Rauen (69) the methylene blue reduction with plant-seed extracts and glycerophosphate was accelerated greatly by the addition of flavoprotein and cozymase.

Lehmann & Mårtensson (70) studied the fall of oxygen consumption observed in experiments using old succinic dehydrogenase solutions; this drop was eliminated by repeated freezing. After freezing, the methylene blue reduction was almost identical before and after the experiment; without freezing destruction was observed. Since, parallel to the increase in stability, an increase in catalase activity with the used enzyme preparations could be shown, the authors discuss

the possibility that the enzyme destruction may be due to hydrogen peroxide accumulation.

Thunberg (71) studied the methylene blue reduction brought about by homologous primary alcohols with extracts from different seeds. Methyl alcohol did not follow the other members of the series. In many cases this alcohol was inactive in spite of a considerable activity with the others. However, in some cases methyl alcohol was also active. The possibility of a special enzyme was discussed. The same author (72) described a dehydrogenase that is present in plant seeds and is specific for glycogen and starch. Thunberg (73) reported that the presence of phosphate is necessary for the dehydrogenation of hexosediphosphate with plant-seed extracts.

Reichel & Neeff (74) prepared an enzyme solution from liver which decomposed citric acid anaerobically. In their scheme the hydroxyl and carboxyl groups which are bound to the same carbon atom are supposed to react with each other, whereby formic acid and acetone dicarboxylic acid are formed. The acetone dicarboxylic acid is then decarboxylated. The reaction was entirely uninfluenced by the presence of oxygen, or of methylene blue, or flavin. No loss in activity was found after dialysing for fifteen hours, and the addition of a cozymase preparation had no effect. Wagner-Jauregg & Rauen (75) studied the dehydrogenation of citric acid with phosphate extract from cucumber seeds. Methylene blue reduction as well as oxygen consumption were greatly enhanced by the addition of flavo-protein and of cozymase. Isocitric acid showed the same activity as citric acid; per mol of acid, one atom of oxygen was taken up. Acetoacetic acid or acetone were not detected.

Das (76) purified by adsorption amino acid dehydrogenase from kidney. The preparation contained no cozymase, and the addition of pyridine nucleotides had no effect. A pronounced activation was brought about by addition of half the volume of heated and filtered enzyme solution. Yeast was found to contain great amounts of an activator which has been purified by adsorption. Webster & Bernheim (77) studied the dehydrogenation and deamination of the isomers of various amino acids with *Bacillus pyocyaneus*. Potassium cyanide inhibited the oxygen consumption strongly. No increase was observed with pyocyanin and methylene blue.

Stone *et al.* (78) measured the methylene blue reduction with resting propionic acid bacteria, using a potentiometric method. The activity of various fatty acids was investigated. The greatest activity

was found with propionic acid; acetic acid followed, while formic acid showed a slight activity. No reduction took place with butyric and valeric acids. Of the dicarboxylic acids, succinic showed the highest reducing power; fumaric and malic acids were active to a lesser degree. The hydrogenation of double bonds by yeast and yeast extracts has been studied by Fischer & Wiedemann (79). Only a double bond in the $\alpha:\beta$ -position to a hydroxyl or carbonyl group was found to be attacked. More double bonds in the same molecule, even if conjugated to the $\alpha:\beta$ double bond, were never hydrogenated.

Work on the interesting "Stickland reaction" has been continued by Woods (80). The reaction is a coupled oxido-reduction between pairs of amino acids brought about by strict anaërobes (*Clostridium*). Ornithine was found to act as a powerful hydrogen acceptor. Together with alanine acting as donator, it was reduced stoichiometrically to δ -aminovaleric acid and ammonia. Alanine was oxidised to pyruvic acid and ammonia. The δ -aminovaleric acid was isolated and identified. By replacement of the α -amino group by a hydroxyl, an entirely inactive product was derived.

Stern (81) demonstrated that by the action of catalase on monoethyl hydrogen peroxide, aldehyde and other substances unidentified up till now were formed. He concluded that the function of catalase might not be confined to the decomposition of hydrogen peroxide; this was also indicated by the work of Keilin & Hartree (82). The latter authors made it probable that ethyl alcohol is oxidised to aldehyde by catalase and nascent hydrogen peroxide. The nascent hydrogen peroxide could be derived for this oxidation from enzymatic oxidations yielding hydrogen peroxide, and from peroxides attacked by catalase.

HYDROGENATION WITH MOLECULAR HYDROGEN

It had been shown that formic acid is decomposed into carbon dioxide and hydrogen by *Bacterium coli*. Recently Woods (83) demonstrated that in bicarbonate solution under an atmosphere of hydrogen with 5 per cent of carbon dioxide, the reverse reaction is catalysed by the same bacterium. Under these conditions hydrogen is rapidly absorbed, and bicarbonate is reduced to formate. By different means it could be shown that the same enzymatic system catalyses both the decomposition and the synthesis; in other words the enzyme forms a reversible system with the substrate. The equilibrium concentration of formate—that is the concentration at which, with fixed concen-

trations of sodium bicarbonate and hydrogen, hydrogen is neither absorbed nor evolved—has been determined. From this value the equilibrium constant, and the free energy for the reaction, $\text{H}_2 + \text{HCO}_3^- = \text{HCOO}^- + \text{H}_2\text{O}$, has been calculated. The values for the free energy calculated from the experimental data and from known free energy data agree within the margin usually found in such calculations. The mean between the two values lies at $\Delta F_0 = -500$ cal. The great interest of this work lies in that this, or a similar enzymatic system, might be involved in the process of photosynthesis.

A powerful activation of molecular hydrogen by *Acetobacter peroxidans* was shown by Wieland & Pistor (84). The bacteria alone absorbed hydrogen to a certain extent; addition of methylene blue increased the hydrogen absorption, and the dye was rapidly reduced. Oxyhydrogen gas (*Knallgas*) was absorbed with great velocity and water was formed. The bacteria oxidised alcohol to aldehyde and acetic acid, but in contrast to the other acetic acid bacteria they did not contain catalase. As Wieland showed, no hydrogen peroxide could be detected after respiration, but hydrogen peroxide was easily reduced by alcohol.

THE ACTION OF DINITROPHENOLS

The mechanism of the action on metabolic processes brought about by these compounds has been greatly clarified by the work of Krahl & Clowes (85). Using sea-urchin eggs, they showed that compounds in which the nitro groups were replaced by halides had entirely the same effect as the nitro compounds. Both greatly accelerated the respiration in very low concentrations, and at the same time blocked the development of fertilised eggs. Both effects were reversible. Significantly, the cessation of cell division appeared in most cases with concentrations slightly exceeding the optimum concentration for the effect on respiration [Clowes & Krahl (86)]. Of the polyhalophenols the 2,4,6-triiodophenol was the most active. It was ten times as active as 2,4-dinitrophenol. The action of this group of compounds is interpreted as being specific for a phenolic hydroxyl in the same benzene ring with substituents imparting strongly negative properties to the molecule. No effect of halophenols on rat metabolism was found and the same was true for tissues *in vitro* examined at body temperature. At lower temperatures, also with halophenols, a certain effect was found, however.

Ronzoni & Ehrenfest (87) described the release of metabolic functions in resting muscle brought about by dinitrophenol. In respiration, as well as in glycolysis, the values found approach those for excited muscle. A very similar effect produced by the action of methylene blue on resting muscle had been found by Gerard (88).

ACTION OF DYES AND THE PASTEUR EFFECT

Hoogerheide (89) studied the inhibition of anaërobic fermentation brought about by indophenols. Five different dyes were tried using yeast suspensions. The dyes inhibited strongly while the leuco compounds had little or no effect. If the dye concentration was so balanced that during the remaining fermentation the dye could be entirely reduced, the fermentation rose almost to the normal level; the fermenting cell was shown to be able to reduce a dye which inhibits in the oxidised but not in the reduced form. Similar experiments had been made by Lipmann (90), using glycolysing and fermenting cell extracts. Lennerstrand & Runnström (91) described progressive inhibition of fermentation in maceration juice by pyocyanin and methylene blue, in air but not in nitrogen.

Michaelis & Smythe (92) made a comparative study of the action of a number of dyes on fermentation by yeast maceration juice. The effect was found to be due chiefly to the individual nature of the dye, and independent of the potential range. As a rule the leuco compounds were not at all or less inhibitory. A large group was found to promote respiration, in the course of which, fermentation was gradually and irreversibly destroyed. Another group, comprising naphtholsulfonateindophenol, rosinduline GG and brilliant alizarin blue, gave an interesting form of fermentation, viz., glucose alone was not fermented, but with hexosediphosphate in the presence of glucose a mixed fermentation took place which yielded large amounts of hexosemonophosphate besides alcohol and carbon dioxide.

In the course of an investigation of the inhibitory action of various dyes on invertase, Quastel & Yates (93) made some interesting experiments with methylene blue. The dye effect on invertase was shown to be due to a reversible ionic reaction between the enzyme and the dye ion. After adding to methylene blue a reductant which was able to reduce the dye in the absence, but not in the presence, of oxygen, an inhibition could be shown which was ultimately due to the reoxidation of the leuco dye with oxygen. The same type of experiment had been carried out by Lipmann (90) to show the inhibitory action

of oxygen on extract fermentation and glycolysis. He assumed that the action of the dye was due to an oxidation of part of the fermenting system. Quastel's experiment shows that such an inhibition is not necessarily due to an oxidation of the enzyme.

In the anaërobic cell, part of the cell's own catalysts, working as hydrogen carriers to oxygen, are entirely reduced. The fundamental change brought about by the presence of oxygen is the oxidation of those catalysts. This change can be described, not inadequately, as an increase in the potential of the catalysts. There is reason to assume that changes in the metabolic function of the cell are associated with the transition from the anaërobic to the aërobic state, as the Pasteur effect should depend on the state of oxidation of those catalysts.

Dickens (94) studied the metabolism of tumour and normal tissues in the presence of various oxido-reduction indicators. With certain reservations he states that strongly positive compounds like ferricyanide, Bindschedler's green, and toluylene blue cause a fall in aërobic glycolysis of tumour tissue. Very pronounced effects on aërobic glycolysis were described with pyocyanin (inhibition), and phenosaphranine (acceleration). With phenosaphranine in concentrations of 10^{-5} *M* the Pasteur effect was entirely abolished in brain and, in somewhat higher concentrations, also in tumour tissue. Other dyes having as low or lower potentials were without effect. In a study of a number of substances chemically related to phenosaphranine, Dickens found the inhibition of the Pasteur effect with compounds containing a quaternary nitrogen in a phenazine or pyridine ring and additional basic groups in the molecule.

Ashford & Dixon (95) had found that in brain suspended in 0.1 *M* KCl-Ringer aërobic glycolysis rises to the anaërobic level. Disturbance of the ionic equilibrium of the cell interior was assumed to be the cause of this effect. In accounting for the observation that such a disturbance brings about a glucose disappearance greater or equal to that found in anaërobic cells the mechanism of the Pasteur effect was discussed by Dixon (96).

Kluyver & Hoogerheide (97) determined with the platinum electrode the potentials in suspensions of metabolising organisms. Small amounts of permeable indicators were used as mediators. Values of *rH* between 8.6 and 9.6 were found with six different kinds of yeast when fermenting anaërobically. With eleven different kinds of lactic acid-forming bacteria *rH* values between 4.9 and 5.8 were obtained. From the great uniformity of potentials found with organisms of a

certain metabolic type, it was concluded that the values obtained are characteristic for the respective processes. Aërobically, with different yeast types, the potential was found to depend upon the ratio of respiration to aërobic fermentation.

ASCORBIC ACID; SULPHYDRYL COMPOUNDS

Barron, DeMeio & Klemperer (98) studied very carefully the auto-oxidation of ascorbic acid. In a solution freed from all traces of metals, no auto-oxidation was observed up to a pH of 7; from pH 8 upwards a rapid irreversible auto-oxidation took place. Copper in concentrations as low as 10^{-6} *M*, and hemochromogen were catalytic. The oxidation with copper as catalyst was completely reversible up to pH 5. Borsook & Jeffreys (99) observed that ascorbic acid could be reduced by a great excess of glutathione. At pH values above 5.5, oxidised ascorbic acid underwent an irreversible change. This change was shown to be a transformation, and not a further oxidation, which proceeded as well in the absence as in the presence of oxygen.

Hopkins & Morgan (100), in a fine study, clarified the mechanism of the catalytic oxidation of glutathione by ascorbic acid oxidase plus ascorbic acid, first observed by Szent-Györgyi (101). The enzyme solution which catalysed the oxidation of ascorbic acid was the juice of the florets and central white stalks of cauliflower. Glutathione was not oxidised by the enzyme, but in the presence of ascorbic acid, glutathione was oxidised preferentially; the rate was the same as that for ascorbic acid alone. Anaërobically, oxidised ascorbic acid was reduced rapidly by glutathione, but only in the presence of the enzyme. The rate of reduction was five times that of oxygen consumption. It was pointed out that ascorbic acid acts here as the coenzyme which enables the enzyme to oxidise glutathione. A protein from liver was isolated which protects glutathione against catalytic oxidation with copper. Zilva (102) reported that ascorbic acid oxidase from apple juice acts as well upon *d*-glucoascorbic acid as upon the natural ascorbic acid. The *d*-glucoascorbic acid had been found to be entirely inactive as vitamin C. It was concluded that the enzyme is not structure specific.

Ball (103) reported that reliable potentials with ascorbic acid are only measurable in the presence of a mediator such as copper, iron, or methylene blue. The value $E_0 = 0.392$ was found under these conditions.

Kharash *et al.* (104) studied the catalysed oxidation of thioglycolic acid, especially in regard to the action of copper. Muscle and sarcoma extracts inhibited iron catalysis, but accelerated copper catalysis, while liver extracts were without effect on iron catalysis, but inhibited copper catalysis.

Schubert (105) described in detail the fairly rapid reaction between iodoacetate and tertiary amines. He (106) studied further the combination products of sulphhydryl compounds and aldehyde or pyruvic acid.

Linderstrøm-Lang & Duspiva (107) found that the digestion of wool keratin by the larvae of clothes moths is made possible by a preceding reduction of the -S-S-groups of keratin in the intestine of the larva. The SH-keratin, only, is attacked by the proteolytic enzymes.

Waddington, Needham & Brachet (108) found that methylene blue is able to induce the gastrula ectoderm of amphibia to form nervous tissue. Cresyl blue and dinitrophenol were found to be inactive.

Lemberg & Wyndham (109) showed that in metabolising tissues, biliverdin could be reduced to bilirubin.

Wurmser *et al.* (110) measured the potentials in a mixture of oxidised and reduced reductone. The potentials became constant after twenty to forty hours. The value of 0.282 volt for E_0 was obtained from these measurements.

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ENZYMES*

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ENZYMES INVOLVED IN BIOLOGICAL OXIDATION AND REDUCTION

GENERAL SURVEY

Great progress has taken place during the past year in the study of enzymes involved in biological oxidation and reduction. Papers have appeared which have thrown considerable light on the basic problem of enzyme chemistry: the chemical constitution of enzymes. Although a preliminary note appeared in 1935 on the constitution of catalase, the detailed paper by Stern (1) from 1936 must be mentioned here. He found significant evidence for the very important fact that the "prosthetic" group of catalase is identical with the protohematin in hemoglobin. If this be accepted, it would seem that for the first time we have a clear example of an enzyme the prosthetic group of which is of known constitution, and the specificity of which is solely determined by its colloidal part, the protein component. It is very likely though that in the not-too-distant future another "active" group in catalase will be sought for, i.e., the part of the protein constituent involved in the combination with the hematin. After all we may regard the protein as being an enzyme capable of catalyzing the reaction between the hematin and hydrogen peroxide, although the formal advantages of this conception are not as obvious in this case as in the case of the dehydrogenases discussed below.

It is also very important to note the spectroscopic demonstration of a catalase-substrate complex formed by the enzymatic reaction [Stern (2)].

Another heme compound, cytochrome-*c* (from ox heart), was purified by Theorell (3). It was not obtained in crystalline form but showed properties which indicated that it was nearly homogeneous. (Isoelectric point 9.82 to 9.86; 0.34 per cent of iron; molecular weight 16,500.) The absorption spectrum was investigated between 220 and 640 μ and it was found that the spectrum of the oxidized cytochrome depends on the pH of the solution, three spectroscopically different forms being distinguishable.

* Received February 9, 1937.

As regards the other important oxidative system in the organism, the flavin system, significant investigations were carried out by Kuhn on the constitution of flavin-enzyme (flavoprotein). Kuhn, Rudy & Weygand (4) synthesized cytoflav (lactoflavin-5'-phosphoric acid). By combining this with Theorell's protein (5), Kuhn & Rudy (6) were able to show that catalytically active flavoprotein was formed. Also with dimethyl-9-*l*-araboflavin phosphoric acid and Theorell's protein a highly active substance was made [Kuhn, Rudy & Weygand (7)]; the authors point out the interesting aspect of preparing other active protein compounds from vitamin-like substances of the flavin group.

Michaelis, Schubert & Smythe (8) made an extensive study of the redox potentials of flavins and came to the conclusion that in the physiological pH range, at 50 per cent reduction, 10 per cent of the dyes are present as radicals (semiquinoid form). This may be of the greatest interest for the reaction between flavin enzyme and cytochrome (see below) and for the enzymatic properties of the flavin enzyme in general. The author regrets being unable to enter more deeply into this interesting paper. Reference may be made to measurements of Kuhn & Boulanger (9) who found a great change in redox potential when the flavin phosphoric acid was linked to the protein. Due to this peculiar fact they suggested that two linkages are involved in the combinations of cytoflav with the protein, the phosphate group and the imino group of the alloxan ring.

Concerning other properties of the flavin enzyme it may be mentioned that Kekwick & Pedersen (10) made an ultracentrifugal study of flavoprotein and found a molecular weight of 80,000, which corresponds to one molecule of flavin per molecule.

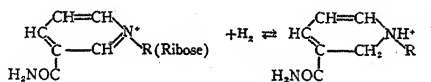
In its relation to cytochrome-*c*, flavoprotein behaves qualitatively as it does to methylene blue [Theorell (47)]. The reduced form of flavoprotein may be oxidized by cytochrome. Theorell assumes that in cells containing the cytochrome system the dehydrogenation of reduced flavoprotein may be effected by cytochrome and not by auto-oxidation. Flavoprotein on the other hand reacts with the reduced forms of the important pyridine compounds which have been shown recently to play an important rôle in fermentation, glycolysis and, although non-autooxidative, by their reaction with the flavin system, also in respiration. Through the fundamental work of Warburg it has been known for some time that pyridine catalysts are involved in certain biological oxido-reductions [Warburg & Christian (11)]. The great

progress of the past year resides in the identification of a basic constituent of cozymase with nicotinic acid amide [Euler *et al.* (12, 13, 14, 16, 17); Warburg & Christian (15, 18)]. Cozymase, therefore, is a hydrogen carrier closely related to the coenzyme of the Robison-ester dehydrogenase [Warburg & Christian (11)], the main difference being that the latter has one more molecule of phosphoric acid in its molecule than the former. Warburg & Christian (18) name the two carriers diphosphopyridinenucleotide (cozymase) and triphosphopyridinenucleotide.¹ It may be possible that in the cell the one is transformed into the other.

Schlenk & Euler (19) suggest the following constitution for the cozymase:

Nicotinic acid amide-ribose-O · (P : O) · O · (P : O) · O-ribose-adenine. According to Myrbäck (20, 21) the properties of cozymase indicate the presence of an enol-lactone in the molecule, but how this may be reconciled with the above formula is not clear.

The mechanism of enzymatic dehydrogenation is that the substituted pyridine group receives two molecules of hydrogen from the donator and gives them off to the acceptor:

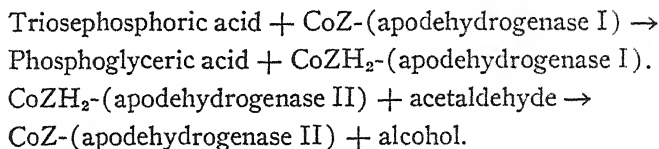


The reduced form shows a white fluorescence and a characteristic absorption band at 340 mμ [compare Warburg & Christian (11)] by the appearance of which the progress of dehydrogenation of the cozymase may be followed. There is some indication that an intermediate semiquinoid form exists [Karrer & Benz (22); Adler, Hellström & Euler (23)]. The nonenzymatic reduction of cozymase may be effected by sodium hydrosulphite in pure solutions of cozymase, but the enzymatic reduction by hydrogen donators such as alcohol requires certain colloidal substances, by Warburg called *Zwischenfermente* or simply "proteins," by Euler [see, e.g., (13)] "apodehydrogenases." The conception is that the cozymase, as a sort of prosthetic group, by combining loosely with these substances, forms different dehydrogenases ["holodehydrogenases" (13)] in the same way as was assumed to be the case with the Robison-ester dehydrogenase [Nege-

¹ In the following paragraphs these nucleotides will be referred to as DPN and TPN, respectively.

lein & Haas (24)]. In fermentation the cozymase (CoZ , CoZH_2) is assumed to assist in the following hydrogen transport:

Scheme I



From this it appears that if the holodehydrogenases are regarded as the enzymes, their specificity is solely determined by their colloidal parts, the proteins, and the connection with the above named catalase work of Stern becomes apparent. However, as the holo-enzymes do not act as genuine catalysts in the half-processes of Scheme I, it would seem more satisfactory in this case to regard the apodehydrogenases as the enzymes, e.g., as catalysts capable of accelerating the bimolecular reaction between donator and coenzyme or coenzyme and acceptor, and to use the name transporter or carrier for the coenzyme. Even if it is shown that part of the protein action is a one-sided activation of the carrier (e.g., a change of equilibrium or rate of transformation between its normal and semiquinoid forms) one must take account of the specific activation of the substrates as well. For this reason the extreme position taken by Negelein & Haas (24) that the *Zwischenferment* is no real enzyme is not likely to be generally accepted. It may even be pointed out that it is possible, though not likely, that in the dehydrogenation mechanism an oxido-reduction of the apoenzyme may take place. The evidence brought by Negelein & Haas in disfavor of this conception is inconclusive.

While touching upon the subject of coenzymes acting as hydrogen carriers it is necessary to call attention to the interesting possibility pointed out by Lipmann (25) that aneurin, vitamin B_{12} , might have a similar function in the cell. Through the work of Peters, aneurin was shown to be of importance in the metabolism of pyruvic acid [compare Peters (26, 27)]. The presence of a quaternary ammonium group in its molecule, and its reduction by hydrosulphite—features which it has in common with the pyridine carriers—are very suggestive [see also Krebs (28)].

A very instructive example of hydrogen transport was given by Hopkins & Morgan (29). They found that addition of glutathione protects ascorbic acid against oxidation by oxygen and ascorbic acid

GROUP 2

Anaërobic	x \searrow dye or x \searrow unknown carriers \searrow dye
Aërobic	x \searrow O ₂ (H ₂ O ₂ formed) or x \searrow cytochrome \searrow O ₂ or other carriers
Peroxidative	x \searrow H ₂ O ₂ .

The dehydrogenases of the first group are best known. Negelein & Gerischer (32) have prepared a protein of globulin character with its isoelectric point at pH 4.82. Upon addition of TPN and flavo-protein it catalyzes the aërobic dehydrogenation of Robison ester to phosphohexonic acid (uptake of 1 atom of oxygen per molecule). Negelein (33) described another protein which he named "A" that plays an important rôle in fermentation [compare (18)] where it reacts with DPN and either alcohol or triosephosphoric acid. Papers by Green & Brosteaux (34), Green (35), Adler & Michaelis (36), Euler, Adler & Kyrning (17), Euler, Adler & Hellström (16), Warburg & Christian (37) also may be mentioned in this connection. It appears from this group of workers that the lactic and malic dehydrogenases of mammals, and the triosephosphoric acid and alcohol dehydrogenases of yeast, all belong to Group 1 and react with the pyridine carrier, DPN (cozymase), while the dehydrogenases from yeast, involved in the dehydrogenation of phosphohexonic acid (37), seem to react with TPN; compare here Dickens (38) and Lipmann (39). The glucose dehydrogenase from liver was reported by Das (40) to employ both carriers. This may be due to the presence of an enzyme capable of transforming one carrier into the other.

As regards citric dehydrogenase from liver, Reichel & Neeff (41) reported that neither DPN nor the flavin system seems to be involved in the aërobic process, though Wagner-Jauregg & Rauen (42, 43) found flavoprotein active (under aërobic and anaërobic conditions) in the case of the corresponding enzyme from cucumber seeds. The oxidation products were quite different in the two cases, and entirely different enzyme systems seem to be involved.

A similar divergency is found between the action of α -glycerophosphate dehydrogenase from rabbit muscle [Green (44)], and cucumber seeds [Wagner-Jauregg & Rauen (45)]. This emphasizes the danger of drawing conclusions from one enzyme system and applying them to another. In this connection a paper by Reichel & Eckhoff (46) may be mentioned in which it is reported that propionic

aldehyde dehydrogenase reacts aëroically with flavoprotein, cytochrome, and indophenoloxidase [compare Theorell (47)].

As regards the enzymes of the second group attention should be drawn to papers by Keilin & Hartree (48, 49) and Dixon & Keilin (50) concerned with the enzymes uricase (from pig's liver), amino acid dehydrogenase (from kidney), and xanthine dehydrogenase (from milk). Apart from interesting features of the relation of these enzymes to inhibitory substances, they brought out an interesting point of view concerning the fate of the hydrogen peroxide formed by the aërobic oxidations in which these enzymes are involved. It is assumed that hydrogen peroxide, when not decomposed by catalase, *in statu nascendi*, by the help of a peroxidase or even catalase itself (?), may transfer oxygen to other oxidizable substances such as alcohol, thus causing an induced oxidation. A similar view is held by Wieland & Pistor (51) in the case of the alcohol dehydrogenase from *Acetobacter peroxidans*. This type of coupled reaction may eventually turn out to be of great importance in metabolism.

Of other enzymes in Group 2, proline oxidase [from pig's kidney, Das (52, 53)], ascorbic acid oxidase, and the phenol oxidases may be mentioned. Tyrosinase action on cresol and catechol was studied carefully by Wagreich & Nelson (54) who found some interesting reactions with aniline in the latter case. An elaborate study of laccase was carried out by Suminokura (55). Sumner & Howell (56) prepared peroxidase from fig sap and found that the substance presumably contained a reduced hematin compound. Tauber (31) found the interesting fact that ascorbic acid is readily oxidized by peroxidase if substances capable of forming quinones are present. These are reduced by ascorbic acid and in turn reoxidized by peroxidase.

Finally the author may call attention to the interesting enzyme or enzyme system, hydrogenase, present in *Acetobacter peroxidans* and capable of oxidizing molecular hydrogen aëroically or anaëroically to hydrogen peroxide [Wieland & Pistor (51)].

MISCELLANEOUS ITEMS

Catalase.—Purification of catalase: Stern (1), Keilin & Hartree (49), Barkan & Schales (57); activation of catalase from body fat by heating to 40°: Bodnár & Bártfai (58); inactivation of mussel catalase by glutathione and ascorbic acid: Marks (59); inactivation of catalase by oximes treated with acid: Sevag & Maiweg (60); inactivation of catalase by ultraviolet light: Yamafuji (61, 62).

Indophenoloxidase, cytochrome, flavoprotein.—Indophenoloxidase in milk inhibited by hydrogen cyanide: Bigwood & Thomas (63); indophenoloxidase in

eggs of grasshoppers: Bodine & Boell (64); the Actinians containing a mixture of heme compounds among which cytochrome-*b*, and *a*, and a "free hematin" may be demonstrated: Roche (65); cytochrome of the heart of horse: Roche (66); synthesis of flavins: Karrer *et al.* (67, 68); splitting of lactoflavinphosphoric acid by α -glycerophosphatase: Rudy (69).

Pyridine carriers.—Reduction of methyliononic acid amide by hydro-sulphite: Karrer & Warburg (70), Warburg & Christian (18); preparation of pure DPN: Euler, Albers, Schlenk & Günther (71), Euler, Albers & Schlenk (12); chromatographic separation of DPN and TPN in yeast; Euler & Adler (13); separation and preparation of DPN and TPN in red blood cells: Warburg & Christian (15, 18); analysis of DPN and TPN: (12, 14, 15, 18); *d*-ribose phosphoric acid from DPN: Schlenk (72); inactivation of DPN by kidney phosphatase: Myrbäck & Oertenblad (73); liberation of nicotinic acid amide from DPN by heating in alkaline solution: Schlenk & Euler (74).

Dehydrogenases.—Inhibition of dehydrogenases from cucumber seeds by tannin: Thunberg (75); necessity of the presence of inorganic phosphate for the dehydrogenation of hexosediphosphoric acid by methylene blue and enzymes from different plant seeds: Thunberg (76); malic dehydrogenase in cucumber seeds: Thunberg (77); reaction of adrenaline with lactic dehydrogenase: Green & Brosteaux (34); dehydrogenation of polyvinylalcohol: Dammann, Lange, Bredig & Nord (78); influence of sodium fulminate on anaërobic and aërobic dehydrogenation of alcohol: Wieland, Ranch & Thompson (79); dehydrogenation of isocitric acid by dehydrogenase from cucumber seeds: Wagner-Jauregg & Rauen (43); glycerophosphate dehydrogenase from muscle of rabbit, using methylene blue technique, non-inhibition by potassium cyanide or sodium azide, slight inhibition by sodium fluoride or iodoacetate, cytochrome believed to be its natural hydrogen acceptor under aërobic conditions: Green (44); carbon monoxide inhibition of fatty acid dehydrogenase from *Lupinus albus*: Craig (80); non-inhibition of proline oxidase from pig's kidney by glutathione, ascorbic acid, and hydrogen peroxide: Das (52, 53); inhibition of ascorbic acid oxidase from the press juice of the pods of the drumstick tree by hydrogen cyanide and hydrogen sulphide; Sreenivason (81); *d*-gluco-ascorbic acid oxidation in apple juice: Zilva (82); purification of uricase: Truszkowski & Guberman-owna (83); pH optimum of uricase, 8.8, (83); amino acid oxidase reacting better with oxygen than with methylene blue, no carbon monoxide inhibition: Keilin & Hartree (48); amino acid oxidase in relation to different substrates: Kisch (84, 85); uricase reacting only with oxygen, no carbon monoxide inhibition (48); non-inhibition of xanthine dehydrogenase by carbon monoxide, hydrogen sulphide, and pyrophosphate; slow, irreversible inactivation by cyanide; inhibition by sodium azide + hypoxanthine: Dixon & Keilin (50); identity of Schardinger enzyme and xanthine dehydrogenase: (50), Michlin, Jemelmanow & Solowjewa (86); inhibition of Schardinger enzyme by cozymase: Andersson (87, 88); non-reactivation of copper-poisoned xanthine dehydrogenase by pure cozymase (89); cyanide inhibition of xanthine dehydrogenase from liver—a hydrogen peroxide inhibition due to poisoning of catalase: Roberts (90); allantoinase in air-living arthropods: Rocco (91).

Hydrogen lyase from *Bact. coli* catalyzing the reversible process $\text{HCOOH} \rightleftharpoons \text{H}_2 + \text{CO}_2$: Woods (92).

Tyrosinase.—Only those phenols likely to produce *o*-quinones forming pigment with tyrosinase: Nobutani (93); with ascorbic acid no inhibition of tyrosinase when acting on tyrosine: Schaaf (94); pH-optimum 6 to 7, substrate cresol (54).

Peroxidase.—Coenzyme of peroxidase from germ of *ricinus* seeds; Garilli (95); inhibition of peroxidase from potatoes by hydrogen cyanide: Tamai (96); aniline peroxidase from horse radish: Mann & Saunders (97); adsorption of peroxidase to cellulose: Tauber (98).

SPECIAL ENZYMES ENGAGED IN GLYCOLYSIS, FERMENTATION, OR OTHER METABOLIC PROCESSES

GENERAL SURVEY

The enzymes of this group are of various types and would be more conveniently taken up in the concluding part. However, some of the results reported are closely connected with the preceding and following parts and may be mentioned here. Giršavičius & Heyfetz (99), Nagaya, Yamazoye & Nakamura (100), and Yamazoye (101) described a peculiar reaction between glyoxalase, methylglyoxal, and glutathione which is important in the mechanism of the activation of glyoxalase by glutathione. By the catalytic action of glyoxalase a compound between glutathione and methylglyoxal is formed and may be isolated (101). This compound undergoes a slow spontaneous decomposition into glutathione and lactic acid, but the decomposition may be accelerated by glyoxalase. A product formed from methylglyoxal and glutathione, without the intervention of glyoxalase, is different and decomposes spontaneously into the original substances.

Meyerhof, Lohmann & Schuster (102) described an aldolase present in muscle and yeast. It catalyzes the reaction: Glyceric aldehyde + dihydroxyacetonephosphate \rightarrow *d*-fructose-1-phosphate, and other reactions of this type. Phosphorylation seems to be required. The enzyme does not seem to be identical with Neuberg's carboglycase which, according to some workers, is responsible for the acetoin formation [compare Tomiyasu (103)]. It may be a part of the zymohexase system [compare Bauer (104, 105, 106)] which catalyzes the reaction: Hexosediphosphate \rightarrow 2 dihydroxyacetonephosphate.

According to Albers & Schneider (107) carboxylase is activated in two ways: by the Auhagen coenzyme + phosphate, and by adenylic acid or heat-inactivated cozymase and phosphate + magnesium ions.

Jacobsohn & Soares (108) reported the interesting fact that aspartase preparations from bacteria or pig's liver catalyze the addition of hydroxylamine and hydrazine to fumaric acid. Crotonic acid does

not react. According to Pereira-Forjaz & Jacobsohn (109) fumarase activity is somewhat lower in D_2O than in H_2O .

MISCELLANEOUS ITEMS

Racemization of *d*- or *l*-lactic acid by enzyme systems present in *Clostridium butylicum* and related microorganisms: Tatum, Peterson & Fred (110); prevention of copper-poisoning of zymohexase by special substance prepared from muscle extract or by cysteine, cyanide, and glutathione: Bauer (104, 105, 106); carboxylase in Swedish top yeast: Wülfert (111); non-decarboxylation of *o*-quinolinepyruvic acid by carboxylase: Neuberg & Minard (112); poisoning of carboxylase by aromatic sulphonic acids: Axmacher & Ludwig (113); decarboxylation of methylethylpyruvic acid, non-decarboxylation of trimethylpyruvic acid: Fromageot & Chantemesse (114); carboxylase models: Langenbeck & Triem (115); fumarase in cucumber seeds: Thunberg (77).

PHOSPHATASES

GENERAL SURVEY

As in the case of the dehydrogenases the transport of phosphoric acid in the metabolic system is connected with carriers. At least two seem to be involved: adenylic acid and diphosphopyridinenucleotide (DPN, cozymase). The action of the latter is not yet quite clear but in several cases it seems to be able to replace the former, which was shown to be active in a great number of phosphorylations and dephosphorylations, i.e., transport of phosphoric acid from one compound to another; these processes may be called "trans-esterifications" (*Umesterung*).

The following chain of processes is typical for the trans-esterification in the metabolic system:

SCHEME II

2 Phosphopyruvic acid + adenylic acid \rightarrow

2 pyruvic acid + adenylypyrophosphate.

Adenylypyrophosphate + glucose \rightarrow

adenylic acid + phosphorylated glucose.

or, written as in case of the dehydrogenases,

x (phosphopyruvic acid) \rightsquigarrow adenylyl carrier \rightsquigarrow y (glucose).

Another typical process is

x (phosphopyruvic acid) \rightsquigarrow adenylyl carrier \rightsquigarrow H_2O
(formation of inorganic phosphoric acid).

That DPN is able to replace adenylic acid may be due to the great similarity in structure. This similarity is such that it may even be that adenylic acid is split off from DPN or that DPN is synthesized from adenylic acid by enzymatic processes in the cell.

At the present stage it is impossible to classify the phosphatases according to their carriers as was attempted in the case of the dehydrogenases. It may even be regarded outside the scope of this review to give examples of processes in which DPN and adenylic acid are assisting [compare Parnas (116); Parnas & Ostern (117); Ostern, Guthke & Terszakowec (118)]. Many of these processes are probably not pure phosphorylations but are coupled with oxidative or reductive reactions. As it is unlikely that the trans-esterification passes through inorganic phosphoric acid it seems obvious from Scheme II that interest must be centered on two kinds of phosphatases: enzymes catalyzing the bimolecular reaction between substrates and carriers, and enzymes involved in the splitting off of phosphoric acid from the carriers.

However, just as in the case of the dehydrogenases, a great number of phosphatases exists the action of which is unlikely to be contingent upon carriers of the above type, and which, therefore, may be assumed to work with other carriers or without carriers, i.e., $x \sim H_2O$.

As an example of the first group "heterophosphatase" may be mentioned. It is active in the second half-process of Scheme II. According to Bauer (119), it is neither identical with Albers' top yeast phosphatase, nor with α -glycerophosphatase. Glucose may be replaced by other acceptors.

The second group comprises several enzymes the chemistry of which is but little clarified: adenylypyrophosphatase [Jacobsen (120); Satoh (121); Haase (122)], DPN phosphatase [Myrbäck & Oertenblad (73)], pyrophosphatase [Bauer (123, 124)], etc. The latter enzyme is activated by magnesium ions, has a pH optimum of 6.6 (123, 124) and is distinct from adenylypyrophosphatase which does not attack pyrophosphate and is not activated by magnesium ions.

Regarding most of the phosphatases, one may distinguish between two groups, (a) those with acid pH-optima, inhibited by fluoride, not affected by magnesium ions [compare, e.g., Pfankuch (125)], and (b) those with alkaline pH-optima, activated by magnesium ions, and not affected by fluoride. Since in fermentation the phosphorylation processes are activated by magnesium ions and inhibited by fluoride [compare, e.g., Lennerstrand & Runnström (126, 127, 128)] it is

apparent that both types of phosphatases are involved in metabolic processes.

Schäffner & Krumei (129) [compare also (130, 131)] studied the phosphatases of yeast. They distinguish between their α -glycerophosphatase (bottom yeast phosphatase) with an optimum pH of 6.4, the top yeast phosphatase of Albers & Albers (132) with an optimum pH of 4 and broad in specificity, the Bauer pyrophosphatase, and the phosphatase of Schuchardt (133, 134) with an optimum pH of 9.

Bamann & Salzer (135, 136, 137) have been engaged in studies of taka phosphatase and the phosphatases of mammals. The pH dependency of the taka-phosphatase activity towards the two substrates, β - and α -glycerophosphate, was investigated before and after treatment of the enzyme with dilute ammonia. From their results they concluded that two "acid" phosphatases are present with pH-optima of about 4 and 6.2. On the other hand considerations by Courtois (138), connected with experiments of the same kind, led him to abandon the idea of a duality. He found that the form of the pH curve was highly dependent upon the substrate concentration, the two optima moving towards one another when this was increased. The experiments by Courtois are quite convincing but naturally more material must be compiled before the matter may be regarded as clarified. In the case of mammals, Bamann & Salzer found similar results. They distinguished between four different enzymes, "alkaline" phosphatase (e.g., bone phosphatase), the phosphatase in red blood cells [compare Schoonover (139)], and two "acid" phosphatases present in tissues.

Kutscher & Wörner (140) prepared a very pure prostata phosphatase with a pH optimum around 6; it is an "acid" phosphatase.

As regards the more complex phosphatases, the nucleotidases, it may be mentioned that an enzyme of this type is in emulsin (141, 142); guanylic acid and thymonucleic acid are attacked.

Finally Pereira-Forjaz & Jacobsohn (109) report that phosphatase activity is only slightly inhibited by D_2O .

MISCELLANEOUS ITEMS

Complex phosphatases.—DPN (cozymase) is phosphorylated by phosphopyruvic acid: Meyerhof & Kiessling (143).

Phosphatases.—Micromethod for determination of "alkaline" phosphatase in blood: Lundsteen & Vermehren (144); preparation of bone phosphatase: Lora Tamayo & Segovia and Lora Tamayo & Blanco (145, 146). Phosphoglyceric acid is split to phosphoric acid and pyruvic acid by washed human placenta,

Antoniani & Clerici (147). Phosphoglyceric acid, hexosediphosphoric acid, Robison ester and phosphopyruvic acid are split by "alkaline" phosphatase in yeast: Schuchardt (133, 134). Phosphotartaric acid is attacked by yeast, muscle, and kidney phosphatases: Neuberg & Schuchardt (148). Brain phosphatases, pH optima, 5.3 and 9.2: Cedrangolo (149), Giri & Datta (150, 151); activation of the "alkaline" phosphatases of bone, kidney, and intestine by α -amino acids and magnesium ions: Bodansky (152, 153); failing correlation between blood and milk phosphatases during lactation: Folley & Kay (154); phosphatase of human milk: Giri (155); distribution of phosphatase in rat intestine: Westerbrink (156); distribution of phosphatase in different plants and seeds: Ignatieff & Wasteneys (157); poisoning of prostata phosphatase by narcotics: Kutscher & Wörner (140); influence of citric acid and related substances upon taka phosphatase: Bamann & Salzer (158).

Nucleotidases.—Nucleotidase from organs of cat, dog, and rabbit: Ishikawa & Komita (159); resistance of nucleohistone against nucleophosphatase: Schmidt (160, 161); nucleophosphatases in snake venoms: Contardi & Ravazzoni (162).

Polydiamino phosphatase.—Thannhauser & Reichel (163).

OTHER ESTERASES

GENERAL SURVEY

Just as in the case of the dehydrogenases, we find theories advanced for the esterases which ascribe the power of determining the specificity within a narrow group of "isodynamic" enzymes to the colloidal part of their molecules, while the group specificity still is believed to be determined by the prosthetic part. The evidence brought forward in favor of this point of view is meagre, but as a working hypothesis it may prove of value and eventually it may even turn out to be the truth. Kraut & Pantschenko-Jurewicz, who call the colloidal part "pheron" and the prosthetic group "agon," have centered their efforts on the separation of these two enzyme constituents; previous papers [compare (164)] seemed to indicate that a separation is possible. Bamann & Feichtner (165), in trying to verify the identity of the "agon" of pancreas and liver esterase required by the theory of Kraut & Pantschenko-Jurewicz, made what would seem to be a very clear-cut experiment. Pancreas and liver esterases show a distinct difference in their attack on the stereoisomers of mandelic acid methylester. This property must be ascribed to the respective pherons. Hence the union of the agon of liver esterase with the pheron of pancreatic lipase would result in a formation of true pancreas lipase recognizable by its relation to the above substrate. However, due to the very confusing results reported later on by Bamann *et al.* (166, 167), the negative result obtained loses much of its strength as an argu-

ment against the theory of Kraut & Pantschenko-Jurewicz. It seems as if the optical specificity of these enzymes is a matter of such complexity, that nothing definite can be said about it at present.

In the further progress of their work, Pantschenko-Jurewicz & Kraut (168) communicated some new experiments on liver esterase. They found the Tillmann reaction to be positive in the case of the active enzyme, but negative with the enzyme inactivated by dialysis against 0.01 *N* HCl. From this, and from the fact that inactive esterase may be made slightly active by addition of ascorbic acid, they conclude that the agon (the smaller diffusible part) is a compound similar to ascorbic acid.

Bamann & Rendlen (169) have given some new data concerning the substrate specificity of esterases. They point out that the reason why substrates like succinic acid monoester are so very slowly hydrolyzed by esterases may be sought for in the presence of the electrically charged carboxylate group in the molecules. The effect of this group may be abolished by amidation, etc., or by increasing the acidity of the reaction medium, viz., by transforming the carboxylate groups into uncharged carboxyl groups.

The scientific harvest of the year comprises a number of papers dealing with choline esterase, the new esterase so important in physiology. Kahane & Lévy (170, 171) studied the action of this enzyme on different derivatives of acetylcholine. They found the interesting fact, that the ethylester of betaine is hydrolyzed, and that eserine does not seem to inhibit the reaction in this case. If this result is reproducible it would indicate the presence of another enzyme. On the basis of inhibition experiments with prostigmine, Easson & Stedman (172) made some interesting but rather uncertain calculations of the number of butyrylcholine molecules split, per active enzyme group per second, under their experimental conditions. Quastel, Tennenbaum & Wheatley (173) made an important study of the synthesis of acetylcholine in brain slices and suggested a connection between glucose metabolism and ester synthesis.

Another case of ester synthesis was reported by Itoh (174, 175). According to this author, a crystalline substance may be prepared from *Ricinus* seeds which when present in the oxidized form is capable of accelerating the enzymatic synthesis of esters. In the reduced form it retards synthesis and accelerates hydrolysis by esterases. Since this would involve a displacement of the point of equilibrium between ester, alcohol, and acid and since no energy-delivering processes are

mentioned, this interesting finding cannot be accepted until further evidence is brought forward.

Neuberg & Cahill (176) made an extensive study of the scission of chondroitin-sulphuric acid by sulphatases from different sources.

MISCELLANEOUS ITEMS

Lipase in the California sea mussel: Fox & Marks (177); esterase in eggs of cephalopods: Kamachi (178); lipase in secretion from dog intestine: Pierce, Nasset & Murlin (179); synthesis by lipases: Sym (314, 315); activation of pancreas-lipase preparations upon standing: Bamann & Feichtner (180); non-splitting of substrates like cholesterol stearic acid ester by *Ricinus* lipase: Reichel & Reinmuth (181); activation of pancreatic lipase by cyanide, inhibition by dihydroxyphenols: Weinstein & Wynne (182); inhibition of choline esterase by morphine and apomorphine: Bernheim & Bernheim (183); choline esterase in different organs: Quastel, Tennenbaum & Wheatley (173); in paramecium: Bayer & Wense (184); esterase models: Langenbeck & Baehren (253).

CARBOHYDRASES

GENERAL SURVEY

The question concerning the specificity of carbohydrases is still in a state of flux. The attractive theory of Weidenhagen seems to lead to exactly the same conclusions concerning the constitution of enzymes as were characteristic of the prevailing points of view regarding other enzymes discussed above. We have to distinguish between differences in group specificity (α -glucosidases, β -glucosidases, etc.) and specificity differences within the groups. The individual variations within groups become apparent mainly when enzymes of the same group specificity but from different sources are compared. When in two polemic papers Hofmann (185, 186) turns against this point of view, it is easy to follow him in his wish for greater simplicity, but there is probably no alternative to a resigned acceptance of the situation. It is of course possible that the classification of Weidenhagen is not complete and that other factors determining group specificity will have to be considered, but if the protein part of the enzymes is conceded to have the power of regulating specificity, it must be expected that specificity to a certain degree will depend upon the species, or the organ, or the cell from which the enzyme is prepared, and also upon the method of preparation.²

A number of specificity determinations were carried out by several

² As regards the state of enzymes in the cell, and the series of complex processes involved in the liberation and removal of an enzyme from a cell, the reader may be referred to a recent paper by Willstätter & Rohdewald (187).

authors. Weidenhagen & Renner (188) emphasize that α -glucosidase and α -galactosidase are two different enzymes because top yeast rich in α -glucosidase is entirely free of α -galactosidase. Since melibiose and α -phenyl galactoside are split with velocities the ratio of which varies with the enzyme preparation applied, they suggest that two different α -galactosidases exist. It is a question, however, whether the specificity depending upon source may be explained so simply. Veibel *et al.* (189, 190, 191) made careful studies of the hydrolysis of many substrates by emulsin. Helferich & Weber (192) found that emulsin was unable to split off the disaccharide, as such, from glycosides of cellobiose or maltose. According to Helferich & Vorsatz (193) the emulsin from coffee beans is different from the enzyme in almonds in that it contains a β -*d*-galactosidase.

Caldwell & Doebbeling (194) have succeeded in separating α - and β -amylase of barley malt by fractional precipitations with ammonium sulphate and alcohol.

Freeman & Hopkins (195) studied the enzymatic breakdown of starch and its derivatives by α - and β -amylase. They confirmed the older experiments by Kuhn and Ohlsson. α -Dextrins and, more slowly, α -maltose are formed by the action of α -amylase, while β -amylase splits off β -maltose and leaves a starch-like carbohydrate unattacked. According to Myrbäck (196) the hydrolysis of starch by α - and β -amylase is not influenced by oxidation of the starch molecule with hypiodite. Blom, Bak & Braae (197) made an extensive study of the separate and simultaneous actions of different purified α - and β -amylase preparations on starch from potatoes. On the basis of a rather simple conception of the simultaneous action of two enzymes upon the same substrate, they came to the interesting and somewhat astonishing conclusion that one-fourth to one-fifth of the glucosidic linkages in the starch molecules are split as well by α - as by β -amylase.

As regards activation and inhibition of carbohydrases, Weidenhagen & Lu (198) found that β -*h*-fructosidase and amylase are inhibited by ascorbic acid. The inhibition is abolished by thiol- and S-S compounds, amino acids, and citrate [compare Hanes (199)]. Osmic acid has a similar effect on emulsin, as found by Helferich & Vorsatz (200). The action of dyes on invertase was studied by Quastel & Yates (201) who suggest from their experiments that this enzyme acts as a *zwitterion*, the positive group combining with the fructose part of saccharose, the negative with the glucose part.

The mode of activation of the inactive β -amylase in the barley grain was the subject of a discussion between Myrbäck & Myrbäck (202) and Chrzaszcz & Janicki (203, 204, 205). The former assume that the indisputable effect of substances like hydrogen sulphide or hydrogen cyanide is an activation of the barley proteases which, in turn, liberate the enzyme in an active form. Chrzaszcz & Janicki hold the opinion that the proteolysis is of no importance for the activation of the amylase, and use as argument that no parallelism exists between the rate of activation and rate of proteolysis. Although Myrbäck & Myrbäck are right in denying the absolute value of this argument, the demonstration of such a parallelism would be the only convincing proof of their theory. Since Chrzaszcz & Janicki give convincing evidence of a reversible activation of soluble amylase by hydrogen sulphide, we may conclude that, although proteolysis may play a rôle in making the amylase soluble, another more direct activation takes place. All authors agree that the other amylase in barley, α -amylase, is not activated together with the β -amylase. According to Giri & Sreenivasan (206) and Chrzaszcz & Janicki (207, 208) α -amylase is present in the early stages of the ripening of the grains of rice, rye, oats, wheat, and barley but becomes inactive as the ripening proceeds. As is well-known it regains activity during sprouting.

Regarding synthesis and equilibria brought about by carbohydrases, we may mention the careful work on β -glucosidase by Ionescu & Kizyk (209, 210) and especially by Veibel & Eriksen (211, 212). Their results are interesting from an "enzymatic" point of view in that they show the reversibility of enzyme catalysis in this case.

Fox & Craig (213) studied the amylase activity in heavy water. As found for other enzymes (Pereira-Forjaz & Jacobsohn), the effect of D_2O is but small.

Finally Ehrlich, Guttman & Haensel (214, 215) continued their studies in the field of the pectinases. In *Penicillium Ehrlichii* they distinguish between a protopectinase, which makes water-insoluble pectin soluble, a pectolase attacking pectolic acid (a complex tetra-galacturonic acid), and arabanase which splits arabane to *l*-arabinose.

MISCELLANEOUS ITEMS

Dilatometric method for carbohydrase determination: Sreerangachar (216); microdetermination of carbohydrases: Heatley (217); β -glucuronosidase in the spleen of bulls: Oshima (218); α -glucosidase (maltase) in livers of different animals and in sera of rat and ox: Glock (219); carbohydrases in the California sea mussel: Fox & Marks (177); carbohydrases in Onychophora: Heatley

(217); adaption of *Saccharomyces cerevisiae* to galactose no result of natural selection: Stephenson & Yudkin (220); special polysaccharase from pneumococcus: Dubos & Bauer (221, 222); nucleosidases in organs of cat, dog, and rabbit: Ishikawa & Komita (159); cerebrosidase: Thannhauser & Reichel (163).

Amylase.—In eggs of cephalopods: Kamachi (178); in silk worms and larvae of other butterflies: Yamafuji, Hiraiwa & Goto (223, 224); in rat saliva: Cohn & Brooks (225); in rabbit saliva: Thomas (226); in secretion from dog intestine: Pierce, Nasset & Murlin (179); in cabbage: Rubin & Trupp (227); in maple sap: Bois & Nadeau (228); in wheat: Ugrumov (229); taka amylase: Kitano (316); glycogenase from rabbit liver: Hodgson (230); glycogenase from liver of different animals: Glock (231); action of ultraviolet light on amylase: Banerjee & Sen (232); non-effect of short electric waves upon amylase: Kosieradzki (223); action of halogenides upon pancreatic and salivary amylase: Clifford (234).

PROTEASES AND AMINO-ACYLASES

GENERAL SURVEY

A great number of crystalline enzymes are found among the individuals of this group and consequently the knowledge of their constitution is more developed than in other cases. So far no success has followed attempts to separate a prosthetic group from the rest of the molecule. It is likely that the linkage involved is very strong and that when broken it cannot be reformed in a reversible manner. It seems, therefore, that the enzymes are comparable to the apo-enzymes mentioned in the first section of this review in that they are proteins, they catalyze a bimolecular reaction (here between peptide bonds and water), and they still need a hundred years of interesting work to have their constitution elucidated.

The admirable preparative work on pancreatic enzymes by Kunitz & Northrop was published in detail this year (235). They describe the preparation of crystalline trypsinogen, trypsin, a trypsin inhibitor, and a compound of this inhibitor and trypsin. Much of the material was presented previously in short notices, but the beauty of the work is more evident from this detailed description. The trypsin inhibitor seems to be polypeptide in nature and possessed of a molecular weight of 6,000. It combines with trypsin in the pH range 7 to 3.5; at pH 1 the compound dissociates.

In continuation of the work by Holter & Northrop (236), Herriott & Northrop (237) describe the preparation of crystalline pepsinogen. The substance is activated in acid solution by an autocatalytic process [compare (236)]. Calvery, Herriott & Northrop (238) studied

the amino acid content of crystalline pepsin. In accordance with the acidic properties of pepsin the figures for the basic amino acids are very low (arginine 2.7, histidine 0.05 per cent), those for the dicarboxylic acids comparatively high. However, the most remarkable result is the high tyrosine content (10.3 per cent), the significance of which is emphasized through the work of Herriott (239), who made it likely that acetylation of the phenolic groups of the tyrosine in pepsin causes a decrease in activity.

Steinhardt (240) made an extensive physicochemical study of the inactivation of crystalline pepsin in solution. He suggested that a pentavalent negative pepsin ion is the unstable particle.

As regards the specificity of proteinases the old question about diketopiperazines as constituents of proteins, and consequently as substrates for proteinases, has been taken up again by Japanese workers [Ishiyama (241); Tazawa (242)]. They maintain that diketopiperazines with acid side chains (aspartic acid anhydride) are split by trypsin, while diketopiperazines with basic side chains (like diaminopropionic acid anhydride) are split by pepsin. However, Waldschmidt-Leitz & Gärtner (243) and Greenstein (244) were unable to reproduce their results and the theory will probably have to be dropped once more. Anyhow it would not fit well with the experimental results of Waldschmidt-Leitz & Kofranyi (245) who in studying the splitting of clupein by several enzymes came to the conclusion, previously anticipated by Linderstrøm-Lang (246), that pancreatic proteinase attacks the peptide bond between two arginine molecules in clupein. Other results which support the assumptions of a peptide chain structure for proteins are found in the important papers by Bergmann *et al.* (247, 248, 249, 250, 251). In papain, they found convincing evidence for the presence of a proteinase-like peptidase (or peptidase system) capable of splitting substrates like hippuryl|amide, carbobenzoxyglycyl|glycylglycylglycine, gelatine, and several others (the vertical lines indicate the point of attack). The enzyme is inhibited by phenylhydrazine, a property which serves to distinguish it from other proteinases in papain.

An ultracentrifugal study directly concerned with the first attack of papain-HCN on egg albumin was carried out by Annetts (252). Two types of molecules are formed, a heavy fraction, the molecules of which are of the same size as those of egg albumin itself, and a very much lighter fraction. Annetts assumes that the first action of papain is a "loosening" of bonds within the molecule whereby a "modified"

molecule is formed. Since, however, no control experiments on the action of hydrogen cyanide alone are reported, this effect cannot with certainty be ascribed to the enzyme.

An interesting case of activation was described by Sato & Hirano (254, 255). They confirmed the old observation by Delezenne that snake venoms activate trypsinogen, and found that the activation is a time reaction proceeding with optimal rate at pH 7 to 8. Maschmann & Helmert (256) found that the pyridine carriers are not able to reactivate copper-poisoned cathepsin.

In regard to peptidases, Greenstein (244) continued his interesting studies of the splitting of synthetic substrates by peptidases. Bergmann & Fruton described a prolylpeptidase which splits substrates like glycylprolin, the peptide bond of which is not capable of enolization [compare (257)]. Frankel (258) reported an enzymatic splitting of peptide bonds in bile acids. Johnson, Johnson & Peterson (259) gave convincing evidence that the leucylpeptidase of pig's intestine (260) is activated by magnesium ions. Tsuchiya (261, 262, 263) reported that snake venoms from different species increase the activity of peptidases. The activation was very pronounced and the results could be reproduced with enzymes from several sources and of different purities. A purification of the active principle of the snake venoms was attempted. Mounfield (264) reported the peculiar fact that the dipeptidase of sprouted wheat is activated by cyanide.

The problem of the function of hydrolyzing enzymes in the cell was studied by Holter (265) who made an important investigation of the distribution of dipeptidase in the eggs of several marine invertebrates. He was able to show that the greatest portion of this enzyme is distributed evenly in the matrix, i.e., cytoplasm deprived of the nucleus and all visible granules (mitochondria, etc.). In the case of this enzyme, therefore, these granules do not seem to play any direct rôle in the enzymatic processes of the cell.

Turning now to urease, Grabar & Riegert (266, 267) made a fractional ultrafiltration of urease and of urease digested by trypsin. They confirmed the results of Sumner that crystalline urease is an individual substance and that the action of trypsin, while forming smaller molecules, destroys the urease activity at the same time. Fearon (268) suggested that urease attacks the enolic form of urea, $\text{HO} \cdot \text{C} : (\text{NH}) \cdot \text{NH}_2$, in accordance with the Bergmann theory for peptidase action (257). Smythe (269), in studying the inhibition of urease by iodoacetate and iodoacetamide, found an interesting rela-

tion between the action of these substances on urease on the one hand and on different thiol compounds on the other.

MISCELLANEOUS ITEMS

Proteases.—Micro formaldehyde titrations: Weil (270); assumed presence of a pepsin with pH optimum of 1.8 in yeast: Hecht & Civin (271); protease adsorbed on fibrin: Schmitz (272); papain-like proteinase in *Calotropis gigantea*: Basu & Nath (273); proteinase with pH optimum 8 in snake venom: Sato & Hirano (254); Ghosh (274); proteinases and peptidases in clothes moth and wax moth: Duspiva (275); cathepsin, carboxypolypeptidase, aminopeptidases in trypanosomes: Krijgsman (276); proteases of *Bac. fluorescens liqu.* and *Bac. pyocyaneus*: Gorbach & Pirch (277); cathepsin and peptidase in regenerating tissue of Amphibia: Orechowitsch (278, 279); proteases in *Taenia*: Smorodinzew & Bèbèchine (280); cathepsin in embryo of hen: Mystowski (281); proteases in the California sea mussel: Fox & Marks (177); proteases in sprouting wheat: Mounfield (264, 282); proteases in Onychophora: Heatley (217); peptidase in embryo of hen: Orechowitsch (283); formation of amino acids, especially tyrosine, by peptic digestion of egg albumin and lactalbumin: Calvery (284), Calvery & Schock (285), Calvery, Block & Schock (286), Miller & Calvery (287); peptic digestion of casein: Utkin (288); splitting of edestin and some of its derivatives by pancreatic enzymes: Kiesel & Roganowa (289); action of chymotrypsin and trypsin upon different viruses: Merrill (290); ultraviolet light on pepsin: Banerjee & Sen (291); simultaneous effect of ascorbic acid and different ions on liver cathepsin: Badinand (292); inhibition of proteases in wheat flour by bromate and related substances: Jørgensen (293, 294, 295); non-splitting of dipeptides or tripeptides by pancreatic juice of rabbits: Itzioka (296); splitting of amidated polypeptides by blood serum: Abderhalden (297); case of assumed peptic synthesis of a thyroglobulin-like substance: Salter & Pearson (298).

Urease.—In eggs of cephalopods: Kamachi (178); in seeds of legumes: Sastri & Sundara Iyengar (299); specificity: Bonnet & Razafimahery (300); histological distribution of urease in mucosa of pig stomach: Linderstrøm-Lang and Sjøberg Ohlsen (301).

Arginase.—In eggs of cephalopods: Kamachi (178); activation by managanous ions, splitting of *l*-arginine: Edlbacher & Zeller (302); histological distribution of arginase in rabbit kidney: Weil & Ely (303).

OTHER ENZYMES

Feulgen (304) described a nucleogelase from pancreatin which at pH 6.8 transforms *a*-thymonucleic acid into *b*-thymonucleic acid. The tetranucleotide structure is unchanged and no inorganic phosphorus is split off.

Lundsteen (305) made an elaborate study of the rennin coagulation. The pH optimum for the enzymatic process was found at 5.3. Interesting is his finding that casein may be clotted by the rennin even

in the absence of calcium ions if the pH is chosen near the isoionic point of the protein. A chemical change following the coagulation was not observed with certainty.

The process of blood coagulation was studied by Wöhlisch (306), Fischer (307), Dyckerhoff & Kürten (308), and Chargaff, Bancroft & Stanley-Brown (309). The latter authors investigated the lipids of blood platelets and found that a certain cephalin fraction [compare Wöhlisch (310); Fischer & Hecht (311); Howell (312)] was very active in the clotting of blood plasma. The circumstance that cephalin preparations from other sources were very active, too (while lecithin from yeast was not), indicates that the active principle, the thrombokinase, is not of a very specific nature. A blood-clotting inhibitor was present in defatted blood platelets. Fischer reports that heparin is not able to prevent the clotting of purified fibrinogen by lipids; therefore this clotting seems to be different from that in plasma. According to Dyckerhoff & Kürten calcium is a constituent of thrombin. Wöhlisch, in a review of several interesting papers, suggests that thrombin, the assumed enzyme component in the coagulation system, is a special enzyme, a "denaturase." Whether it has anything to do with the fibrin proteinase studied by Schmitz (272) is uncertain. The interesting work by Fischer (313) suggests a chain-reaction mechanism of the coagulation or an autocatalytic formation of enzyme during the clotting process comparable to the autoactivation of pepsin and trypsin, but it may be pointed out that processes involving formation of new phases do not necessarily need catalysts in the usual sense. The crystallization of a super-saturated solution upon inoculation has features in common with an autocatalytic process; still the crystal seeds added cannot be regarded as catalysts in the usual sense.

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THE APPLICATION OF MICROCHEMISTRY TO BIOCHEMICAL ANALYSIS*

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The applications of microchemistry in biochemical analysis are practically as broad as the field of biochemistry itself; i.e., essentially every major problem of the biochemist has been furthered by the use of micromethods. Therefore, citation of the specific applications is entirely impossible. Much valuable qualitative work and many physical and physicochemical methods must be omitted due to space considerations.

It is unfortunate that, in many instances, methods have been developed with little attention to the chemical soundness or the best available technique. Nevertheless, many real contributions have been made to biochemical progress, and it is probable that here lies the most fruitful field of application of microchemistry. In no other branch of science is the chemistry of traces more important, and perhaps nowhere else are the available amounts of materials so severely limited.

For purposes of discussion in this review, a quantitative microchemical method will be defined arbitrarily as any method in which the amount of material determined is less than a very few milligrams (e.g., 5 mg.). This rather high value is chosen in order to include many of the most useful methods such as the organic combustion procedures for pure compounds. Most of the commonly-used biochemical micromethods involve considerably less than 1 mg. of the material which is to be determined.

In general, it can be stated that microchemical methods require much smaller samples, less time, less laboratory space, much smaller quantities of reagents, and are of about the same accuracy as macromethods.¹ Moreover, they permit many investigations to be carried out which would be impossible or prohibitively difficult or expensive with macromethods as, for example, in the field of histochemistry

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¹ For a discussion of relative costs of microchemical vs. macrochemical laboratory operations, see Kirner (1).

and the allied field of "drop analysis," and in the study of trace elements and trace compounds of physiological interest. The greater ease and rapidity of operations such as ashing, extraction, filtration, etc., make micromethods attractive even when larger amounts of sample are available.

ORGANIC ELEMENTARY ANALYSIS

In 1910, Fritz Pregl, Professor of Medical Chemistry in the University of Graz, started the development of quantitative organic microcombustion methods in order to analyze certain compounds of biochemical interest which he could obtain only in small quantities. From this small beginning grew the monumental methodological studies which today are probably more widely followed in biochemistry than any other type of micromethod. It is not feasible to attempt to list the important and diverse uses of the Pregl methods which have been made in recent years. Many of the biochemical research laboratories of Europe and America depend entirely on these methods for the analysis of the materials isolated from biological systems or synthesized for biochemical use. Much of the knowledge of composition and structure of the hormones, vitamins, oestrogenic and carcinogenic substances, poisons of biological origin, the newer pharmaceuticals, etc., has been obtained by use of the Pregl methods or by modifications of them. As a specific example the work of Wintersteiner, Williams & Ruehle (2) on crystalline vitamin B₁ might be taken.

Carbon and hydrogen.—The carbon and hydrogen method of Pregl (3) has been studied and improved by numerous investigators including Lindner (4), Niederl *et al.* (5), Friedrich (6), Kirk & McCalla (7), and others.

Nitrogen.—Nitrogen is usually determined in pure organic compounds by the Pregl-Dumas method (3). This has been modified by many investigators including, recently, Milner & Sherman (8) and Ide (9). It has been shown that the Pregl-Dumas method does not give correct results with some compounds (8). In these cases the micro Kjeldahl method is of use, as it is also for determination of total nitrogen in biological materials. The method of Parnas & Wagner (10) is most commonly used in these instances. Friedrich, Kuhaas & Schnurch (11) have modified this method for use with all forms of nitrogen except nitrate. Lauro (12) has suggested the use

of selenium as catalyst with a definite shortening of the time of digestion. This has been widely studied and has led to the very useful proposal by Schwoegler, Babler & Hurd (13) of copper selenite as a combination catalyst. Kemmerer & Hallett (14), Kirk (15), and Fife (16) have described improved all-glass distillation apparatus. Friedrich (17) has summarized the developments in the Kjeldahl method up to 1933. Of all individual microchemical methods, the micro Kjeldahl is probably the most extensively used in biochemistry.

Sulfur and halogens.—Sulfur and halogens in organic compounds have been widely determined by the tube microcombustion of Pregl (3) which still remains standard for sulfur determination. A satisfactory volumetric halogen method was introduced by Hoelscher (18), while Leipert (19), Kirk & Dod (20), and others, have profitably applied the Kendall titration procedure to the determination of organic iodine by combustion. Elek & Hill (21) and Beamish (22) introduced micro sodium peroxide bombs for organic combustion for sulfur and the halogens. This procedure is rapid and reasonably accurate but has been used only with a final gravimetric determination.

ANALYSIS OF COMPLEX BIOLOGICAL MATERIALS

Both in the clinic and in the research laboratory, the analysis of biological fluids and tissues for various constituents constitutes the major part of all chemical examinations. While most of the common clinical methods (many of which are colorimetric) are microchemical methods, they are often not so considered. In general, only microadaptations of these standard methods will be included in this discussion. Rappaport (23) has published a complete system of microchemical methods for blood analysis.

The three major trends which have been most useful in opening new biochemical fields for exploration by micromethods are: (a) the development of new sensitive color-forming and precipitating reagents, most of them organic,² such as 8-hydroxy-quinoline (25) and diphenylthiocarbazone (26); (b) the substitution of precise volumetric methods for many of the less accurate colorimetric ones, often with the use of improved volumetric reagents such as ceric sulfate and titanous salts; and (c) the development and improvement of equipment, much of it for physicochemical and colorimetric or

² Cf. Feigl (24) for summary.

photometric analysis. It is convenient to consider these trends under the headings of the constituents which are to be determined.

ALKALI METALS

Sodium.—Accurate microdetermination of sodium in biological materials is dependent on the zinc uranyl acetate reagent of Barber & Kolthoff (27).³ The original gravimetric method is most used, with suitable procedures for avoiding interference, as in the excellent technique of Butler & Tuthill (29) who studied urine, feces, blood, etc. The lower limit of the gravimetric method is about 0.5 to 1 mg. Certain volumetric and colorimetric methods determine much smaller amounts. Salit (30), using a colorimetric modification, studied the comparative sodium content of aqueous and vitreous humor, and serum (31). Oberst (32) similarly studied human red blood cells in pregnant women, and Maizels (33) studied the sodium distribution between corpuscles and plasma. Ball & Sadusk (34) applied the general volumetric procedure of Kolthoff & Lingane to biological materials. Holmes & Kirk (35) modified the method, making it as sensitive as the best of the colorimetric procedures and considerably more accurate. This was applied to the analysis of urine, blood, and stools.

Potassium.—Potassium bears an intimate relation to fundamental cellular processes such as permeability, irritability, and muscle function. It also has a definite pathological significance. Its microdetermination is of interest but is not completely satisfactory. Some modification of the cobaltinitrite method is usually employed; e.g., that of Jendrassik & Takacs (36) which yields a precipitate of reasonably constant composition; that of Rappaport (37) which makes use of a very favorable volumetric procedure; that of Hibbard & Stout (38) which yields greater than usual reproducibility; and that of Robinson & Putnam (39) which is the most sensitive method for small amounts. All cobaltinitrite methods are subject to inconstancy of composition of the precipitate, difficulty of filtration, and the rapid decomposition of the reagent.

The gravimetric determination of potassium as potassium chloroplatinate avoids these difficulties, but volumetric or colorimetric methods of determining the precipitate have been few. Shohl & Bennett (40) converted the chloroplatinate precipitate to iodoplatinate

³ Cf. also Caley & Foulk (28).

which was determined either colorimetrically or volumetrically. Bullock & Kirk (41) titrated the chloride in the precipitate directly. Accurate results were obtained down to 0.2 mg. of potassium. Cahen (42) developed a similar method. Leulier (43) and Cimerman & Rzymowska (44) have reviewed the micromethods for potassium and some phases of its biological significance.

ALKALINE EARTH METALS

Calcium.—The study of calcium in biological systems has proceeded rapidly for some years. Nearly all of the published data on this subject, which have been reviewed by Schmidt & Greenberg (45), were obtained by the use of micromethods. The procedures of Kramer & Tisdall (46) and Van Slyke & Sendroy (47) have been most prominent. Kirk & Schmidt (48) favorably modified the technique of these methods. Greenberg *et al.* (45, 49) used this modification in studies of the forms of calcium in the blood and the relation of the parathyroid thereto. Rappaport (50) and Rappaport & Rappaport (51) improved the calcium oxalate determination by oxidation with ceric sulfate, the excess being determined iodometrically. The latter authors were able to determine calcium in as little as 0.2 cc. of serum. Larson (52) determined excess ceric sulfate with standard ferrous ammonium sulfate and obtained better results; he studied calcium in ashed organs and in serum ultrafiltrates containing down to about 30 $\mu\text{g.}^4$ calcium.

Magnesium.—Micromethods for magnesium in physiological materials depend almost entirely on the use of 8-hydroxy-quinoline (oxine) for precipitation (25). The reagent is quite non-specific but may be used with proper adjustment of the conditions of precipitation and, in most cases, with the preliminary removal of calcium. Yoshimatsu (53) determined colorimetrically the magnesium in 1 cc. of blood, applying the Folin & Denis phenol reagent to the magnesium-hydroxyquinolate precipitate. Eichholtz & Berg (54) modified this procedure advantageously. Bomskov (55) and Greenberg & Mackey (56) determined the precipitate from small amounts of blood by a volumetric bromate procedure. Greenberg and co-workers (57) have made significant studies of the physiology and pathology of magnesium with this procedure. Godden & Duckworth

⁴ 1 $\mu\text{g.}$ = 0.001 mg. = 1 γ .

(58) and Watchorn (59) investigated the physiology of magnesium with the less satisfactory magnesium-ammonium-phosphate method. Cruess-Callaghan (60) recently studied the use of the 8-hydroxyquinoline procedure.

IRON

Iron plays so fundamental a biochemical rôle in connection with respiratory pigments and catalysis that its microdetermination is one of the more important biochemical techniques. This analysis has usually taken one of two general forms: (a) colorimetric, either as ferric thiocyanate or as complexes with certain organic reagents; or (b) volumetric, either by direct titration with a standard reducing agent or by preliminary reduction and re-oxidation with a standard oxidizing agent. In rare cases it has been determined gravimetrically, usually with 5,7-dibrom-*o*-hydroxyquinoline.

The colorimetric thiocyanate procedure has been used most widely. The color is subject to fading through iron reduction by excess thiocyanate (61); is dependent on the concentrations of all reactants and the hydrogen ion (62); and is not completely developed in the presence of many interfering materials (63, 75). Even when extracted with organic solvents there still may be considerable interference (64), especially with very small amounts of iron (61). Barkan (65) and others (66) used this procedure for the study of "easily split" or non-hemoglobin iron of blood. The colorimetric α - α' -dipyridyl method (67) has been favorably reported and applied by Scharrer (68), Kohler, Elvehjem & Hart (69) and others. McFarlane (70) has compared this method with that using titanous titration.

Micro volumetric iron methods can be made as sensitive as existing colorimetric methods with the advantages that interference can be eliminated and the personal factor reduced to a minimum. McFarlane (62) and others (61, 71) have employed titanous salts for the direct titration of ferric iron. When properly performed, this method is quite sensitive and accurate. McFarlane has shown its superiority to thiocyanate microcolorimetry. McFarlane & Milne (72) utilized this method in the study of iron and copper metabolism in the chick embryo. Dubnoff & Kirk (61) determined amounts of iron as low as 2 μ g. in a concentration of 0.5 part per million with an accuracy of about 1 per cent. This procedure was adapted for study of serum and non-hemoglobin blood iron.

Reduction of iron to the ferrous state by a Jones reductor or, better, by liquid amalgams (73), followed by reoxidation with standard ceric sulfate, permanganate, or dichromate, is a simpler technique with somewhat narrower applications (74). Of the various oxidizing agents for ferrous iron, ceric sulfate is undoubtedly the most advantageous.

Stary (75) and Frommes (76) have reviewed the question of iron analysis in biological and other materials.

MISCELLANEOUS HEAVY METALS

At present, only a limited number of heavy metals are of direct biochemical interest, viz., zinc, copper, lead, and a few others. The major progress in determining these metals has consisted primarily in the development of organic reagents giving precipitates (e.g., oxine) or intense colors (24) with the metals in question. In the latter type an outstanding example is dithizone (diphenylthiocarbazone) which yields a strongly-colored solution in chloroform or carbon tetrachloride with those metals mentioned, as well as with various others (77). Its lack of specificity in most instances necessitates a preliminary separation of the metals present. However, the small number of metals encountered in biological materials permits a greater utility in this field. Thus, Tompsett (78) and others (79) have studied the distribution of lead in normal and pathological tissues and fluids. Wichmann *et al.* (80) have reviewed the methods for the determination of lead in foods. Hibbard (81) has developed and applied a very sensitive dithizone method for the study of zinc in plant materials. Deckert (82) applied the reagent differently to the determination of zinc, while Sahyun & Feldkamp (83) have made significant studies of the physiological distribution of zinc, using a less favorable method.

Schultze, Elvehjem & Hart (84) studied the blood, tissue, and organ copper in relation to nutritional anemia, using the colorimetric dithizone method of Fischer & Leopoldi (85). McFarlane (86) applied another organic reagent (sodium diethyldithiocarbamate) to the microcolorimetric determination of copper in the developing chick embryo (72). Tompsett (87) also utilized this method for the study of copper in blood, urine, and feces. Sarata (88) made use of "cryogenin" in a series of investigations of the biochemistry of copper, while Hahn & Fairman (89) used the volumetric chromatropic

(1,8-dihydroxy-2-nitroso-3,6-naphthalene disulfonic acid) method of Ansbacher, Remington & Culp (90). Gerlach and collaborators (91) have made interesting studies on the copper contents of organs of the fetus and adult, and of tumors, using the rather difficult but very sensitive spectrographic method. Many other investigations of the physiology of copper have been made by use of micromethods.

HALIDES

Chlorine.—The high concentration of chloride in biological materials has not been favorable to the general use of microchemical methods for its determination. However, several highly advantageous micromethods have been developed and used. Osterberg (92) applied the adsorption indicator, dichlorofluorescein (93), to the direct semi-micro silver-nitrate titration of blood chloride. This was extended to a full micromethod by Teschan & Kirk (unpublished data) and later by Saifer & Kornblum (94) who used the method for various physiological fluids. They later modified it for use in acid solution (95) with diphenylamine blue as indicator. Rose (96) and Collier (97) have further extended the use of the dichlorofluorescein method. These procedures have proven advantageous in studying large, as well as small animals, because of their ease, accuracy, and rapidity.

Conway (98) has developed a simple and very sensitive micro-diffusion method for chloride in minute amounts of blood, urine, and tissue. It has been shown by Sunderman & Williams (99) that the open Carius digestion for chloride, as used by Van Slyke (100) and others (101), does not give complete recovery. Haslewood & King (102) have recently published an iodometric procedure for chloride which had been previously abandoned in the author's laboratory due to its dependence on temperature and other conditions.

Bromine.—Bromine has been claimed to be of fundamental significance in connection with certain mental disorders, in the production of normal sleep, and in other respects. Due to its low concentration in the organism only micromethods may be used for its determination. Few of these have been satisfactory and many of the suppositions regarding its physiological importance have undoubtedly been based on faulty analytical data (103).

One of the best present methods is that of Conway & Flood (104) who have applied the very useful diffusion technique. The methods

of Leipert & Watzlawek (105) and of Ucko (106) likewise appear to be reasonably dependable. These authors (106, 107) confirmed the secondary physiological significance of bromine. Neufeld (108) has extensively studied the distribution of bromine in both plants and animals. A summary of the literature can be found in the references cited.

Iodine.—Practically all of the more recent knowledge of the distribution of iodine in the animal and plant kingdoms is the result of investigations utilizing microchemical methods. The analytical difficulties due to the low and variable concentration of this element in nature have given rise to a prohibitively large mass of literature regarding both method and application. Thyroid glands (109, 110) and kelp (111) are examples of materials relatively rich in iodine, while blood (112), butter and other fats (113), pasture grass (114) and other animal and human foods (113, 115), and tap water (116) show decreasing concentrations of this element.

The preference for a method involving destruction of the large amounts of organic material which must frequently be used as samples remains a subject of much disagreement. Kendall (117) and Fellenberg (118) first established reasonably satisfactory destruction methods based on oxidation in a caustic melt. Modifications of these original methods are still popular (109), though Fellenberg (119) has more recently abandoned the open caustic fusion and resorted to combustion in oxygen in a closed system. McClendon and coworkers (120), Karns (121), and others have developed similar procedures which are widely used. Hamilton (122) has studied and improved the procedure for extraction of minute quantities of iodide salt. Leipert (123) has more recently developed a much simpler wet digestion procedure which Trevorrow & Fashena (124) have satisfactorily modified in order to eliminate various errors.

The determination of the liberated iodine in the digest or absorption liquid has usually followed the excellent volumetric procedure of Kendall (117), used subsequently by Fellenberg (118, 119), Kelly & Husband (109) and many others. Harvey (125) performed extensive studies on iodine determinations and recommended as a standard procedure a modification of the original Fellenberg method (118) which, he believes, can be reproduced at will. In common with most determinations of traces of iodine, the technique is difficult. Abelin (126) and Fellenberg (127) have reviewed the methods of determination of iodine in biological materials.

PHOSPHORUS

The microdetermination of phosphorus has assumed great importance due to its rôle in connection with normal and pathological bone formation, carbohydrate metabolism, and other physiological functions. There are few significant recent developments. A multiplicity of colorimetric procedures has arisen of which the method of Kuttner & Lichtenstein (128) probably combines the greatest sensitivity and accuracy. The colorimetric procedures are rapid and convenient but are subject to technical difficulties, to various interferences, and to uncertainty with regard to proper reducing agents.

The micromethods for phosphorus which are still the standards for comparison are the gravimetric method of Pregl-Lieb (3) and that of Embden (129). These methods have been replaced in some instances by volumetric procedures such as that of Kuhn (130). Lindner & Kirk (unpublished data) have been able to determine as little as 0.5 ± 0.02 μg of phosphorus by a modified acidimetric method.

Brooke & Smith (131) modified the gravimetric procedure of Pregl for use in mineral nutrition studies (132). Plimmer (133) and Proskuriakov & Temerin (134) studied the technique and errors of the volumetric procedure. A review of the older literature has been published by Feigl, Strebing & Barrenscheen (135).

SULFUR

Sulfur is of biochemical interest in connection with the metabolism of the sulfur-containing amino acids, the relationship of the urinary sulfur fractions to metabolic problems, physiological oxidation, etc. Its accurate microdetermination is still dependent on a gravimetric barium sulfate procedure. For this purpose the simple micromethod of Kirk & Craig (136) is quite accurate and has been found to be satisfactory in biochemical research.

Numerous volumetric and colorimetric sulfate methods have been published. The benzidine procedure, which may be modified for titration or colorimetry, is most widely employed. Among the objections to this method may be listed various interferences, the necessity of standardization of procedure, and the difficulty of obtaining complete precipitation. These errors have been recently studied by Owen (137) with a view to their standardization or elimination.

Barium titration methods, using such indicators as tetrahydroxyquinone (138), are unsuitable for microprocedures (139, and unpublished data). Volumetric and colorimetric barium-chromate methods, such as that of Morgulis & Hemphill (140) and Foster (141), are successful only due to the balancing of relatively large errors, as shown by Manov & Kirk (unpublished data).

NITROGENOUS CONSTITUENTS

Ammonia and urea.—Of the various nitrogenous compounds of living matter, ammonia and urea are of most frequent analytical importance since they constitute the primary end products of nitrogen metabolism of the animal. A very useful new technique for these materials is the application of diffusion by Conway (142). By elimination of distillation or aëration of ammonia, greater accuracy with smaller amounts of material is possible. This technique was applied by Conway (143) to the study of blood ammonia and to urea determination in whole blood, protein-free filtrates, urine, etc.; to urea determination by Rappaport & Gutmann (144); and to determination of ammonia in eggs by Selma & Schaible (145). Other applications to sub-micro quantities are discussed in a later section.

The determination of ammonia in an absorption liquid may be made acidimetrically (e.g., Conway), or by the volumetric or gasometric hypobromite methods (146), or by Nesslerization or other colorimetric procedures (147). Teorell (148) applied the hypobromite method to the determination of 0.001 to 0.02 mg. of ammonia nitrogen in conjunction with distillation. Krogh (149) similarly determined 0.05 to 2 μ g. of ammonia nitrogen in air and water.

In addition to hydrolytic methods for determining urea, it has been frequently determined in small quantities by the dioxanthidyl-urea method. This has usually been applied gravimetrically (150) though Luck (151) and others have modified it for volumetric determination. Wenger, Cimerman & Maultbetsch (152) studied comparatively the gravimetric and the Allen & Luck procedures.

Pucher, Vickery & Leavenworth (153) made careful studies of small amounts of plant ammonia and amide nitrogen by distillation and spectrophotometric Nesslerization. Aëration and Nesslerization are, in general, less satisfactory than the other procedures mentioned.

Uric acid.—The microdetermination of uric acid has not been developed to a satisfactory state. The standard colorimetric method

of Folin (154) is not specific for this material and serves only for a more or less accurate estimation. Investigations such as those of Borsook (155) and Edson & Krebs (156) demonstrate that reasonably simple and dependable methods might be developed without serious difficulty.

Creatine and creatinine.—Creatine and creatinine are in essentially the same state (157), though here also the work of Borsook (155) indicates the possibilities of future investigations of this subject.

CARBOHYDRATES

Reducing sugars.—Most carbohydrate analysis involves ultimately the determination of reducing sugars. Oxidation of the sugar by alkaline copper or by alkaline ferricyanide has been the standard procedure for several years. Many extensions of technique and application have taken place, some of them adapted for microquantities. The advantages of the micromethod involve not only saving in time and expense but the possible use of series determinations, using finger blood or tail blood of small animals; the study of many special biological materials, available only in small quantity, is also possible.

Probably the most important recent advances in reducing-sugar determination have dealt with deproteinization and sample preparation rather than with the actual determination (158). Since no chemical sugar method is specific, the control of interference from reducing non-sugars is of primary importance. Almost without exception the micromethods have depended on ferricyanide reduction by sugar. The most widely used micro sugar method is probably the colorimetric procedure of Folin (159) which is a modification of the Hagedorn-Jensen method (160). Rappaport & Pistiner (161) have directly reduced the scale of the Hagedorn-Jensen method for use with 0.02 cc. of blood. The determination of excess ferricyanide, as in the latter method, is subject to some criticism which is not true of the methods following.

Extending the work of Whitmoyer (162), who developed a cerimetric microtitration method for invert sugar, Miller & Van Slyke (163), Giragossintz, Davidson & Kirk (164), and Vanossi & Ferramola (165) independently developed similar procedures for blood glucose, which depend on the direct ceric sulfate titration of the ferrocyanide formed in the sugar oxidation. Hassid (166) applied the same principle to the determination of reducing sugars and sucrose in

plant materials. These procedures have the advantage of a constant factor relating glucose to ceric sulfate, and a relative independence of heating time and other conditions of the determination. They are applicable to very small amounts of glucose, e.g., that contained in 0.1 cc. of blood,⁵ and are characterized by great rapidity and ease.

While many important studies have been made on reducing sugars in recent years, most of these have not involved the use of methods which are strictly micro and will not be here considered. Dische (167) and Wasitzky (168) have reviewed up to 1934 the micromethods applicable to the determination of various types of carbohydrates in biological materials, while the general considerations of sugar analysis have been discussed at great length in the literature.

Glycogen.—Glycogen has usually been determined by some micro-modification of the Pflüger method (169), i.e., by isolation of the glycogen, followed by hydrolysis and determination of the reducing sugar formed. One of the earliest micromethods was that of Sahyun (170). His use of animal charcoal has been criticized by Good, Kramer & Somogyi (171) and by Simonovits (172), who have developed substitute micromethods. Sahyun has replied to the criticism of Good *et al.*, but does not appear to have answered that of Simonovits. Heatley (173) has described a glycogen method, sensitive to $\pm 2 \mu\text{g.}$, which utilizes about 1 mg. of tissue. He has applied it to the study of glycogen distribution in the regions of the amphibian gastrula in order to determine the degree of glycogenolysis during invagination. Doi (174) and Blatherwick, Bradshaw, Ewing, Larson & Sawyer (175) have developed micro glycogen methods; the latter combine their procedure with the determination of total tissue carbohydrates and lactic acid.

Starch.—Starch is generally determined by hydrolysis, followed by determination of the reducing sugars formed (176), or by isolation and colorimetric determination as starch iodide (177). Disaccharides, such as sucrose (166, 178) and maltose (179), may also be determined by way of the hydrolytic products.

ORGANIC ACIDS

Though organic acids unquestionably play a significant rôle in both plant and animal metabolism, the methods used for their micro-

⁵ Cf. section on drop scale methods.

determination have been far from satisfactory, due to the similarities of reaction with each other and with other related organic compounds. Only a few acids may be determined in biological materials with any degree of confidence.

Lactic acid.—Lactic acid is of primary importance in animal metabolism as a product of carbohydrate utilization. Friedemann & Graeser (180) performed an extensive study of method and application which is perhaps the best recent investigation. Their method was applied to blood, urine, milk, culture medium, and tissues, and was used as a completely micro procedure. Fuchs (181) has developed a very satisfactory type of lactic acid apparatus. Very small amounts (0.05 to 0.45 mg.) have been determined by Hinsberg & Ammon (182) by a modified diffusion apparatus.

Citric acid.—Citric acid has been shown to be a normal metabolic product of both animals and plants. The determination of small quantities has been unusually difficult. The Thunberg procedure (183), using citricodehydrogenase from cucumber seeds, is delicate but unreliable for quantitative use. The pentabromacetone procedures of Pucher, Vickery & Leavenworth (184) and of Pucher, Sherman & Vickery (185) appear to be the most dependable micromethods so far developed. These were applied to urine, excreta, animal tissues, saliva, plant (chiefly tobacco) tissues, and other biological materials.

Oxalic acid.—Entirely reliable methods for oxalic acid seem to be non-existent. Among the better methods is the rather complicated one of Dodds & Gallimore (186), who studied urinary oxalic acid present in fractional milligram quantities.

Malic acid.—Malic acid has been adequately determined in small samples of plant tissues by Pucher, Vickery & Wakeman (187).

Pyruvic acid.—Pyruvic acid has not been satisfactorily determined. The method of Fromageot & Desnuelle (188) yields excellent results in the absence of lactic acid, even on much smaller quantities than specified by the authors. The interference of lactic acid prevents this from being, at present, an adequate method.

Amino acids.—This important group of organic biological materials has received much attention. The literature on the determination of individual amino acids is so voluminous that it cannot be reviewed here. Total amino acids (amino nitrogen) are determined by three types of procedure: (a) gasometric, as in the Van Slyke procedure; (b) colorimetric, as in the method of Folin; and (c) volumetric, in special solvents. The colorimetric methods are subject to

much interference and usually represent only an approximation. The gasometric method is more dependable but necessitates special apparatus and is not trustworthy in the case of mixtures (189). The volumetric methods usually employ alcohol (190), glacial acetic acid (191), or formalin (192), as solvents, generally in combination with some water. These are considered the better methods but are inapplicable to many systems and yield only average values. Since few of these methods have been adapted for microchemical application, they need not be elaborated.⁶ For a critical summary of theory and practice, see Richardson (193) and Van Slyke & Kirk (194). Grassman & Heyde (195) utilized a micromethod for amino acids and peptides and Rosedale & Da Silva (196) for basic amino acids. Cavett (197) developed a small-scale Van Slyke nitrogen-distribution method.

ACETONE BODIES

These materials are of particular significance as incomplete metabolites of fat which remain only in cases of disturbed carbohydrate metabolism, as in diabetes mellitus. Difficulties in their analysis arise partly from interference by other organic materials, and even more from the considerable diversity of the materials classed together as acetone bodies. The most generally used method for their determination is that of Van Slyke which recovers approximately 75 per cent of the total. Edson (198) has modified this procedure for much smaller quantities of material. The method of Hubbard (199) gives better results but the three successive distillations involve a considerable increase in time and manipulation. Hubbard & Wright (200) applied this method in various studies. Robinson & Chaikoff (unpublished data), in studying depancreatized dogs, have been able to combine the triple distillation of the above method into a single operation and obtain quantitative recovery. The application to increasingly smaller amounts of acetone is limited by the inadequacy of all known methods (201) for the final determination of minute amounts of acetone.

CHOLESTEROL

The microdetermination of cholesterol has figured in many biochemical investigations, though the exact function of this material in

⁶ Cf. section on histochemistry, Linderstrøm-Lang & Holter (208).

the animal body is still in doubt. Its determination may be performed colorimetrically by the Liebermann-Burchardt reaction, as in the method of Rappaport & Engelberg (202), or by the Milbradt reaction, as in the method of Gortz (203). It may be more advantageously determined gravimetrically (204) or oxidimetrically (205) as cholesteryldigitonide. In view of the great variability of results obtained by different workers and the uncertainty of the form of much of the cholesterol in the organism, the work of Drecker and coworkers (206) is especially helpful. Wasitzky (207) has reviewed the micromethods for determination of cholesterol.

ULTRAMICROMETHODS

Histochemistry.—In 1931, Linderstrøm-Lang & Holter (208) initiated a completely new scale of microchemical method, adapted to certain determinations of a few gammas ($1 \gamma = 0.001 \text{ mg.}$) or less. By this technique they studied enzyme distribution through the determination of small enzymic cleavages by thin sections of root tips in an appropriate substrate. They extended this technique to include the determination of ammonia and urea (209), arginase (210), sugars (211), and chlorine (212). By the application of these methods they studied peptidase in the root and cotyledon of malt grain, in *Drosera rotundifolia*, in certain marine invertebrates, in the eggs of *Psammochinus miliaris*, and in the stomach and duodenum of hogs (213). In addition they studied the pepsin, acid, and esterase distribution in hog stomach and duodenum as a function of histological structure (214), and the digestion of keratin by larvae of the clothes moth (215).

Levy (216) has developed a method for total nitrogen ($0.5\text{--}6.0 \mu\text{g.} \pm 0.03 \mu\text{g.}$) in successive microtome sections of a grain of barley, using the same fundamental technique. This is similar to the procedure for total nitrogen developed by Borsook (155), discussed below. Weil & Ely (217) investigated the arginase distribution in rabbit kidney by the same technique. Holter (218) studied the peptidase distribution in marine ova, and Glick (219) applied the technique to the determination of lipolytic enzymes. Glick & Biskind (220) further extended these studies to the determination of vitamin C, using the method for the determination of its distribution in various glandular tissues and the small intestine (221).

Drop analysis.—The developments in the field of histochemistry

inaugurated a broader movement to extend the utility of ultrafine technique. The original methods contained certain inherent technical and constructional difficulties and were limited, practically, to the operations of sampling, diffusion, and titration. Kirk (222) published an improved technique for "quantitative drop analysis" in which most of the difficulties of the Linderstrøm-Lang procedures were overcome; methods for quantitatively performing nearly all common laboratory operations on minute samples were detailed. Miller & Kirk (223) applied this technique to the determination of blood calcium in amounts as low as 2.5 μg . Subsequent investigation has shown certain difficulties in this method which have been overcome by Lindner & Kirk (unpublished data) who determined cerimetrically as little as 0.5 μg . of calcium as oxalate. Kirk (224) determined blood non-protein nitrogen by a distillation procedure with as little as 3.88 μg . of nitrogen. Gibbs & Kirk (225) adapted the diffusion method of Conway (142) to the determination of urea and ammonia in samples down to 1.55 μg . Conway (143) later reduced the scale of his method to determine less than $1 \pm 0.02 \mu\text{g}$. for blood ammonia studies. Bentley & Kirk (226) extended the diffusion technique to include the Kjeldahl analysis of total nitrogen. Kirk & Bentley (227) cerimetrically determined from 2 to 15 μg . of iron in blood after preliminary reduction. Lindner & Kirk⁷ (unpublished data) have determined to 0.5 μg . of phosphorus in blood. Heck, Brown & Kirk (unpublished data) have adapted the micro cerimetric procedure (164) for determination of reducing sugars in blood to amounts in excess of 1 μg . All of these methods maintain an accuracy of 1 to 2 per cent.

Borsook (155) described drop-scale methods for determining ammonia, urea, total nitrogen, uric acid, creatinine, and allantoin in amounts of about 1 to 10 μg ., and later applied these to the study of nitrogen metabolism in isolated tissues of the rat (228). This was the first outstanding example of the application of ultrafine analytical methods to the study of metabolism, a central problem of biochemistry. The procedure for the first three materials was based on a combination of the ammonia-diffusion procedure of Conway, with spectrophotometric color comparisons. The remaining constituents were determined by rather direct spectrophotometric procedures modified for use with high dilutions and small amounts of the constitu-

⁷ Cf. section on phosphorus.

ents. Thus, uric acid was first precipitated by zinc, and creatinine was removed for analysis by Lloyd's reagent before the color was developed. The work of Borsook is a more satisfactory demonstration of the utility of the spectrophotometer in small scale analyses than of general drop-analysis technique.

Birch & Harris (229) utilized a capillary burette for the determination of the dissociation constants of hexuronic acid and of vitamin B₁ in 0.1 cc. of solution. Ogston & Peters (230) applied a similar technique to vitamin B₁ and thiochrome.

Capillary tube colorimetry.—An ultramicro colorimetric technique for various constituents of biological systems has been developed by Richards and coworkers (231), and applied to the study of the composition of glomerular urine. Bordley & Richards (232) investigated the uric acid in glomerular urine of snakes and frogs, while Walker & Reisinger (233) studied the reducing substances in similar material from frogs and Necturi. Walker (234) extended the method to the study of inorganic phosphate in the same material, and Bordley, Hendrix & Richards (235) applied it to determination of creatinine. Walker (236) summarized and compared these data with corresponding analyses of aqueous humor, cerebrospinal fluid, lymph, and blood of frogs, higher animals, and man, and concluded that glomerular urine was a simple ultrafiltrate of blood, the other fluids involving additional factors. Westfall, Findley & Richards (237) later adapted Isaacs' chloride method to this technique and found that glomerular urine had approximately the same concentration of chloride as blood plasma.

The methods of capillary colorimetry are necessarily more difficult than the other ultramicro methods which have been discussed. They depend on having very uniform capillary tubing, whose preparation is difficult, though it is now available commercially. All volumes must be determined by micrometric measurements of liquid menisci in the capillary. The final color comparison must be interpolated between fixed standards, similar in principle to the use of Nessler tubes. This, combined with the normal uncertainty of reading small color differences in capillary tubes, involves rather large probable errors. However, its use in such difficult cases is amply justified by the results. Such investigations as these should clarify many of the obscure physiological problems which cannot otherwise be studied due to the scarcity of material.

The past applications of ultramicro technique have merely indi-

cated the possibilities. That entire portion of the biochemical field which deals with small biological systems such as in invertebrate zoölogy, insect physiology, tissue culture and tissue-slice study, embryology, cytology, bacteriology, and protozoölogy should be able to apply it profitably. The advantage of using tail blood of mice and rats for general biochemical studies, thus saving the animal for further use, is obvious. Even the hospital research laboratories, and possibly the clinics, might profitably use ear and finger blood for many purposes, particularly in the field of pediatrics.

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THE CHEMISTRY OF THE CARBOHYDRATES AND THE GLYCOSIDES*

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Polysaccharides.—The endeavour has been made by Staudinger & Eilers (1) to elucidate the constitution of starch by comparing the viscosity and osmotic pressure measurements of solutions of starch dextrans. For the reasons that a starch dextrin always shows, in solvents such as formamide, formic acid, and water, the same specific viscosity, that in any given solvent there is only a small decrease in viscosity with rise in temperature, and that the viscosity of a starch dextrin is the same before acetylation and after de-acetylation, Staudinger holds the view that the starch colloidal particle is a macromolecule rather than a micelle or an aggregate of smaller molecules held together by van der Waal forces. If this view be accepted then the particle weight, determined by some physical method such as the osmotic pressure method, will also be the normal molecular weight. Furthermore, this will also lead to an evaluation of the constant K_m from the viscosimetric relation $\eta_{sp}/C_{gm} = K_m M$. With various dextrans a series of such comparisons led to the evaluation of the constant K_m corresponding to 1×10^{-4} . In these calculations the osmotic pressure figures furnished by Biltz (2) were utilised. Employing a similar analogy to that which led him to establish the K_m value for the cellodextrans and cellulose it is contemplated by Staudinger that this new value of K_m for the starch dextrans which hold for starch itself. From these results it is concluded that the K_m constant for the starch series is about one-tenth of that employed for the cellulose series (10×10^{-4}) and that it is incorrect to use the latter in evaluating the molecular size of starch. From these considerations Staudinger has deduced that the length of the starch macromolecules can only be about one-tenth of that of a cellulose macromolecule of equal molecular weight. This is held to support the view already expressed by other workers that the chain of α -glucopyranose units in starch

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does not lead to a rectilinear or thread-like assemblage but is built up in zig-zag or spiral form with a corresponding diminution in the degree of extensibility of the chain. The physical properties of starch, compared with those of cellulose and of cellodextrins, are also held to be in favour of this view. For example, the insolubility of starch in Schweitzer's reagent is ascribed to the tenacity of the residual valency linkages between the hydroxyl groups of the spiral.

That these ideas are still in a state of flux is apparent from the fact that, shortly following the publication of the paper already cited, Staudinger (3) has found occasion to change the value of the constant K_m for cellulose and this is now given only half the value previously assigned to it. The same author indicates that the viscosity values for the cellulose acetates lead to the same figure for molecular size that has already been published by Haworth & Machemer on the basis of the evaluation of the end group in methylated celluloses (4). It is pointed out by Staudinger that there is now no disagreement between these two methods of evaluation for cellulose acetates and the methyl derivatives prepared from them. Both authors have already indicated, however, that native cellulose must possess a much larger particle size.

In a recent article Haworth (5) has summarised the position in regard to the size of polysaccharide molecules and it is pointed out that the factors underlying these questions can best be investigated by a general study of many types of carbohydrates. It is possible that some common fundamental factor operates in determining the very large size of polysaccharide molecules and that consequently it is undesirable to limit the investigations to the study of one or two members such as starch and cellulose. Starch methylated under a variety of conditions, either directly or through the acetate, may give rise to products of quite varying viscosity. But in all these cases the evaluation of the tetramethyl glucopyranose in the end group furnishes the same value for chain length, namely, 24 to 26 glucose units. This is true whatever may be the source of starch so far as the investigations have proceeded. Up to the present these have included starch from potato, maize, waxy maize, soluble starch, and other prepared starches. It is not considered that this evaluated chain length is to be regarded in any sense as a measurement of the particle size. On the contrary it is believed that these comparatively short chains are linked by co-ordination or by covalent bonds. This may involve not only aggregation to increase the length of the chain but also lateral com-

bination between the chains. These factors must be recognised in any comparison of the molecular weights of cellulose, starch, glycogen, inulin, xylan, or other polysaccharides. It seems, therefore, premature at this stage to crystallise ideas on the subject until investigation from many sides has rendered clearer the interpretations which are to be placed upon any single result.

Other physical measurements for the determination of particle size of polysaccharides have yielded results of great interest and the force of the plea for the exercise of caution in the final evaluation of data will be at once apparent. Using a cellophane membrane Carter & Record (6) have measured the osmotic pressure of acetylated and methylated starches and starch dextrans and of the corresponding derivatives of glycogen, inulin, and other carbohydrates. Two samples of methylated starch from different sources or prepared by different methods showed osmotic pressures corresponding in particle sizes to such high values as 630 and 140 glucose units. The surprisingly large values of 3400 and 5400 glucose units were found in the case of methylated glycogen, although the results of viscosity and end-group assay point to the presence of 12 or 18 glucose residues only in the chain. Using a diffusion method Lamm (7) records a particle weight of 4×10^6 for starch dispersed in zinc chloride while Oakley & Young (8) find that methyl glycogen gives osmotic pressures indicating a mean particle weight of 2×10^6 in dilute calcium chloride solution and of 3.4×10^6 in benzene.

Chowdhury & Bardham (9) report that solutions of cellulose from cotton, jute, and bamboo in Schweitzer's reagent give values by the ultracentrifuge method which correspond to 978, 516, and 189 glucose units respectively; particle weights of the same order are also given by the viscoses prepared from these celluloses. By viscosity methods, and using phosphoric acid as a solvent for Swedish filter paper, Ekenstein (10) finds that the particle weight of this form of cellulose corresponds to 970 glucose units. Continued contact with this reagent degrades native cellulose at a constant rate until the molecular dimensions of hydrocellulose are reached, and thereafter the hydrolysis is much diminished. Degradation is also said to occur by intensive grinding of cellulose in contact with water (11), and after twelve hours the particle size is reduced to a mean value equivalent to 300 glucose units. Further work by Barsha & Hibbert (12) on the celluloses of wood pulp from spruce and maple supports the view that no chemical distinction is to be drawn between the celluloses from wood

or cotton. Wood cellulose in acetone dispersion methylates with ease to give a product containing 44.5 per cent methoxyl and there seem to be no grounds for earlier statements that a portion resistant to methylation exists in cellulose from this source.

From time to time there has been much discussion on the rôle of the phosphoric acid residue in the starches. From potato, sago, and arrowroot the starches yield on acid hydrolysis a glucose-6-monophosphate which, according to Posternak (13), is identical with Robison's ester. The latter product is also isolated by acid treatment of the tetrasaccharide monophosphate prepared by amylolytic hydrolysis of these starches. It is concluded from these and earlier results that the phosphorus is combined in ester form in the starches stored in rhizomes but that it may be present in the form of lecithins in the cereals and not directly linked to the starch.

Mannose polysaccharides, whatever may be found to be their function in plant metabolism, have claimed a new interest in that they frequently occur together with the more widely distributed glucose polysaccharides. In Konjak mannan (14) and possibly in yeast-gum mannan (15) the polysaccharide is composed of glucose intramolecularly united with mannose. But in *Tubera Salep* (16) the mannan (30 per cent) is associated with starch (30 per cent) and a dextrinous product (13 per cent) which is possibly a mixture of mannose and glucose dextrans. The *Salep* mannan, like the A and B mannans of the ivory nut (17), is composed of mannose units mutually linked in the 1:4-positions, as are the glucose units in starch and cellulose. The yield of tetramethyl mannose from methylated *Salep* mannan, following hydrolysis, corresponds to a chain length of about 60 mannose units or a molecular weight of 12,000. On the other hand viscosity measurements of particle size furnish a lower estimate, even taking the minimum value for K_m given by Staudinger for starch. The constant for the mannan series would require to be of the order 4×10^{-5} to contribute the same molecular size as that given by the end-group assay. The *Salep* mannan is fairly soluble in water whilst the ivory-nut mannans are insoluble. This is difficult to reconcile, perhaps, with a low molecular weight. Lateral association of the mannan chains to a degree approaching the spherical form may account for the low viscosities, and such an assemblage may be expected to be less soluble than the free macromolecule.

Considerable interest attaches to the galactose polysaccharides and particularly to the naturally occurring galactans. Meanwhile a

new polysaccharide is reported by Haworth, Raistrick & Stacey (18) which is mainly galactan in character and is prepared by the action of *Penicillium varians* G. Smith on glucose. This carbohydrate is composed of 6 to 8 galactopyranose units linked through 1:4-positions, and terminates at one end by a glucopyranose unit and at the other (reducing) end by what seems to be *d*-idose. A polysaccharide obtained from a red alga (19) appears to be a sodio-sulphuric ester of galactan. It forms diacetyl and dimethyl derivatives and is hydrolysed either by acid or alkali to a galactan. The latter yields a methylated derivative from which a trimethyl galactose, probably the 2:3:6-form, was isolated. It is suggested that the structure of the polysaccharide is that of mutually linked units of galactopyranose united through 1:4-positions and that the sulphuric acid groups occupy the 6-positions in each residue.

Immunopolysaccharides.—A new interest in carbohydrates developed from the observation of Dochez & Avery (20) in 1917 that the pneumococcus, during the early stages of its growth, produces a readily soluble substance which diffuses into the culture medium, and from the demonstration of the polysaccharide nature of this substance by Heidelberger & Avery (21). It is now made apparent from much work carried out in other fields that the formation of specific polysaccharides by micro-organisms is not restricted to pneumococcus. Similar observations have been extended to Friedländer's bacillus (22), tubercle bacillus (23), *Streptococcus viridans* (24), Shiga's bacillus (25), gonococcus (26), meningococcus (27), staphylococcus (28), cholera vibrio (29), typhus bacillus (30), anthrax bacillus (31) and *B. aertrycke* (32). A knowledge of the constitutional forms of the immunopolysaccharides will naturally be of vital interest not only to chemists but to bacteriologists whose activities, once again in the history of chemistry, may run on parallel planes. The experimental work on the chemical and structural sides has been well summarised in a recent paper by Heidelberger, Kendall & Scherp (33). Earlier methods of isolation made use of alkali which, in the case especially of pneumococcus-Type-I polysaccharide, eliminated an essential acetyl group and impaired the active immunising property of the polysaccharide, whilst the characteristic property of precipitating homologous antisera (34) was retained. Modified methods, avoiding heat or strong acid or alkali, are now described (33) for the isolation of the carbohydrates from culture filtrates. Concentration is conducted in a vacuum and the products are re-

peatedly precipitated with alcohol in the presence of acetic acid and sodium acetate. Following the procedure of Sevag (35), proteins are removed by denaturation with butyl alcohol and chloroform, and the starch and glycogen by amylolysis. The final substances are obtained as neutral sodium salts which furnish solutions of high viscosity. These polysaccharides are no longer considered to be heat-stable since heat brings about a decrease of particle size and a reduction in the amount of antibody precipitated from homologous rabbit antisera.

The specific polysaccharide of pneumococcus Type I contains nitrogen, acetyl, and uronic acid groups. On the assumption of a molecular weight of 600 there are present two nitrogen atoms, one acetyl, and two uronic acid groups. About half the nitrogen is free amino nitrogen which is capable of acetylation. Whilst the original product is amphoteric the acetylated material titrates sharply and shows an acid equivalent of 319. None of the nitrogen appears to occur as acid amide and the carboxyl groups of the uronic residues are free. The latter are identified as galacturonic acid by isolation of the crystalline monohydrate of the methyl ester of methyl-*d*-galacturonide from the products of hydrolysis with methyl alcoholic hydrogen chloride (33). It is suggested that the fundamental unit of the large molecule is a trisaccharide composed of two galacturonic acid groups together with an unidentified residue which carries two nitrogen atoms.

The pneumococcus-Type-IV polysaccharide differs from Type I in yielding an amino sugar together with acetic acid on hydrolysis, and thus has some resemblance to chitin. Uronic acid groups appear to be absent. In the polysaccharide from Type II and Type III there appears to be little or no nitrogen. Structural studies with Type III have indicated (36) that the hydrolytic cleavage products are an aldobionic acid, which is present to the extent of 85 per cent, and also glucose to the amount of 9.5 per cent. The fundamental unit appears to be the aldobionic acid, which may stand in the same relation to the polysaccharide as maltose does to starch. This aldobionic acid is monobasic, reduces Fehling's solution, and with hypiodite is oxidised to a dicarboxylic acid giving the naphthoresorcinol test. No mucic acid is found in the product after prolonged boiling with mineral acid and subsequent oxidation and the structure of the aldobionic acid is doubtless to be represented by a glucuronic acid linked through its reducing position to a glucose residue. The hepta-acetate of its

methyl ester is crystalline and seems to be identical with a product derived by a similar procedure from the polysaccharide of pneumococcus Type VIII (37).

Latterly, endeavours have been made to synthesise this and similar aldobionic acids. By condensing 1,2,3,4-tetra-acetyl glucose with the methyl ester of 1-bromo-triacetyl glucuronic acid, Hotchkiss & Goebel (38) have prepared the acetylated ester of 6-glucuronido- β -glucose which is the gentiobiuronic acid derivative. The interconversion to the α -isomeride is achieved by the agency of zinc chloride and acetic anhydride and this also is a crystalline substance. It remains to be decided whether one of these synthetic substances is identical with the product from Type III. By an analogous procedure Hotchkiss & Goebel (39) have also synthesised the aldobionic acid of gum arabic. Its constitution had already been shown to be a 6-glucuronosido-galactopyranose (40).

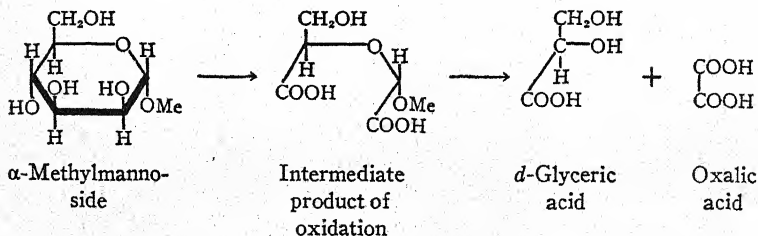
Investigations on the pneumococcus-Type-II polysaccharide have not proceeded far enough to reveal its essential structure (22). It is now known, however, that it leads on hydrolysis to both glucose and a uronic acid (20 per cent).

Some relationship is discernible between the blood-group specific carbohydrates and the above pneumococcus types. Thus the mixture of specific carbohydrates isolated from the urine of individuals of blood-group A has been shown by Freudenberg & Eichel (41) to contain galactose, N-acetyl glucosamine, and a small amount of a uronic acid. Removal of the acetyl group from the carbohydrate results in the loss of specificity, which is regained on re-acetylation. Other workers (42) have examined the polysaccharide of cholera vibrio W.880 and have detected the presence of arabinose, and an aldobionic acid complex of galactose and glucuronic acid. Morgan (25) has given careful study to the *B. dysenteriae* of Shiga from which a specific polysaccharide was isolated, purified, and tested for homogeneity. It appears to be capable of acetylation and benzylation and from these derivatives can be regenerated with alkali, unimpaired both in chemical and immunological properties. The polysaccharide yields on hydrolysis 97 per cent of reducing products, calculated as glucose, and shows an acid equivalent of 9000. Although containing 1.6 per cent nitrogen and 5 per cent acetyl, none of the nitrogen is liberated by nitrous acid. The polysaccharide resembles in this respect N-acetyl glucosamine, and the whole of the nitrogen may occur as the acetylated amino residue. The basal unit

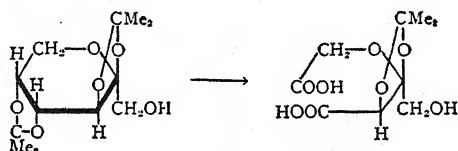
would appear to consist of an N-acetylamino-hexose surrounded by four hexose residues and it is suggested that these groupings are repeated six times in the whole polysaccharide.

Sugars and glycosides.—Under the influence of 0.01 *N* barium hydroxide, *d*-glyceraldehyde is slowly transformed to dihydroxyacetone which then condenses more rapidly with the glyceraldehyde to give both *d*-fructose and *d*-sorbitol, but neither *d*-tagatose nor *d*-psicose. There appears to be a preferential formation of sugars having *trans* hydroxyls at 3- and 4-positions where synthesis has occurred, inasmuch as both tagatose and psicose have *cis* groups at these positions (43). The dihydroxyacetone must engage in aldol condensation at the moment of its formation, since no *dl*-glyceraldehyde appears to be generated. If the enolic transformation be prevented by employing the monoacetone of *d*-glyceraldehyde, then the condensation of two molecules occurs through the aldehyde group of one molecule reacting at the second carbon atom of the other molecule with the production of sugars with branched chains.

Earlier experiments on the oxidation of sugar derivatives with periodic acid (44) resulted in a degradation in, what seemed at first to be, an unaccountable manner. It is now evident that the explanation is more remarkable than could be expected. With α -methylmannopyranoside the successive application of periodic acid and bromine water leads to the complete elimination from the sugar chain of the third carbon atom (45) with complete fission between the second and fourth carbon positions. Similar results follow from the use either of the above reagents or of barium hypobromite followed by bromine acting on pyranoside forms of α -methylglucoside or mannoside. This proof of the tendency of sugars to suffer fission at the second and third or at the third and fourth carbon atoms is of interest biologically. Equally interesting is the implication, evident from the character of the intermediate product and the isolation of *d*-glyceric acid from this reaction, that the ring structure of the normal gluco-



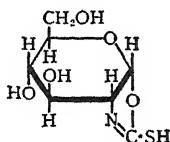
side and mannoside must involve the fifth carbon position in the sugar chain. This conclusion supports the constitutional work on the methylated glucosides and mannosides. Other examples of a similar order of fundamental cleavage of a sugar are furnished by the use of lead tetra-acetate which effects the breakdown of α -methylglucopyranoside to glyoxal, glycollic acid, and a trace only of formaldehyde (46). The same reagent provides a most useful descent of the sugar series from benzylidene arabitol to *d*-threose (47), the latter being readily isolated as its monoacetone. Another example of the utility of the reagent is in deciding in certain cases whether a phosphoric ester of a sugar involves substitution of the primary alcohol position, since formaldehyde is not formed in this event (48). A further type of oxidative cleavage of the pyranose ring is that described by Ohle & Senger (49) in which β -fructose-diacetone is converted in neutral solution to a dibasic acid, as indicated below:



Partly-substituted sugars have been studied in greater detail, among the most interesting being the monophosphates. Phosphorylation of 1,2,3,6-tetra-acetyl- β -glucose leads to the formation of glucose-4-phosphate and this substance has been characterised and its behaviour compared with the 3- and 6-glucose phosphates (50). The 5-methylglucose has been obtained by the following procedure: glucose-diacetone-3-*p*-toluenesulphonate is converted into the corresponding monoacetone derivative from which, by means of benzoyl chloride, 6-benzoyl-3-*p*-toluenesulphonylglucose-1,2-monoacetone is formed. The 5-methyl derivative is then readily formed and, after alkaline hydrolysis followed by acetylation, 5-methyl-3,6-diacetylglucosemonoacetone is obtained. The last substance gives in turn 5-methylglucosemonoacetone, and removal of the acetone residue leaves 5-methylglucose. The question of a possible Walden inversion during removal of the *p*-toluenesulphonyl group is considered by the author and evidence adduced against its occurrence. The final product (5-methylglucose) displays remarkable properties which require further investigation. It reduces Fehling's solution and permanganate in the

cold and appears to exist partially in the aldehyde form since it gives a colour immediately with Schiff's reagent (51). With regard to other monomethyl ethers of the hexoses it is now stated that Pacsu's supposed 4-methylgalactose (52) is really 6-methylgalactose and that this author's claims concerning the corresponding 4-methylmannose cannot be substantiated (53).

A new type of glucose derivative (μ -thiol-glucosaxoline) (Formula I) has been obtained by treating glucose with potassium thiocyanate in the presence of 12 *N* hydrochloric acid. This does not

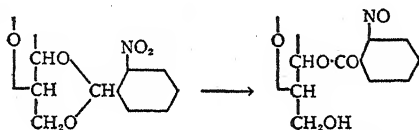


I. μ -Thiol-glucosaxoline

reduce Fehling's solution but appears to form a salt with sodium hydroxide. It is readily decomposed by phenylhydrazine with formation of glucosazone. With alkaline hydrogen peroxide the $-SH$ group is removed and replaced by $-OH$, the resulting substance, μ -hydroxy-glucosaxoline, being now capable of reducing Fehling's solution (54).

The use of acetone derivatives of the sugars as a means of preparing partly-substituted sugars is further illustrated by the recent work of Ohle and his collaborators, who have made a special study of 5,6-anhydroglucose-1,2-monoacetone. The 5:6-anhydro ring can be opened by a variety of reagents and numerous 6-substituted glucoses are made available. Among these may be mentioned 6-thioglucosemmonoacetone, derived by the action of hydrogen sulphide and barium hydroxide on the anhydro body, and from the first named substance 6-thiogluucose is obtainable (55). In addition various sulphides, sulphonic acids and 6-halogenohydrins may be prepared from the anhydro substance. Furthermore, 6-amino derivatives [including, e.g., 6-glucosylpiperidine (56)] are produced when amines are used to open the anhydro ring, and by a similar procedure the 6-phthalimide derivative is obtained (57). Interesting and unexpected results have been encountered in a study of the condensation products of *o*-nitrobenzaldehyde with sugars and glycosides. Glucose, mannose, or galactose gives rise to a di-*o*-nitrobenzylidene derivative, one

of the benzylidene groups being united through the 4:6-position. Whilst these derivatives each contain one hydroxyl group they resist acylation and are otherwise noteworthy in that under the influence of light they isomerise, giving 4-*o*-nitrosobenzoates in place of 4,6-*o*-nitrobenzylidene derivatives. 4,6-*o*-Nitrobenzylidene derivatives of methylglycosides are found to behave similarly. That the high reactivity of the *o*-nitro group is responsible for these transformations is shown by the fact that the corresponding *m*-nitrobenzylidene derivatives are stable to light. The behaviour of these substances appears to be yet further complicated by the occurrence of Walden inversions during the isomerisation, but full understanding of the mechanism involved is at present incomplete on account of the difficulties experienced in removing the nitrobenzylidene and nitrosobenzoyl residues (58).



Evidence of the tendency of mannose-oxime to exist as, or to pass readily into, the open-chain form is furnished by the interesting work of Wolfrom and his colleagues on the aldehyde forms of sugar derivatives. Mannose-oxime forms a hexa-acetate which must be an open-chain compound. By the agency of oxalic acid in methyl alcohol this passes to penta-acetyl-aldehyde-*d*-mannose-oxime which, with nitrous acid, leads to penta-acetyl-*d*-mannose (59). This combines with ethylmercaptan in the presence of zinc chloride giving the same product as is obtainable by acetylation of mannose-diethylmercaptan (60). Further examples of these open-chain products are provided by the hepta-acetates of galactose and mannose, and the hexa-acetates of arabinose, rhamnose, and xylose (60, 61, 63). The stability of these hyper-acetates is characteristic. The hepta-acetate of *dl*-galactose has been isolated as a product of the acetolysis of agar (62). It is doubtful whether this can be considered good evidence for the presence of *dl*-galactose as an open-chain form in agar inasmuch as Micheel *et al.* (61) have shown that a normal derivative of *d*-galactose may be transformed into hepta-acetyl-*dl*-galactose. There seems, however, to be some evidence of the occurrence of

l-galactose in the hydrolysis products of agar. Of very considerable interest are the new types of halogen derivatives obtainable from the acetylated aldehyde forms of sugars, inasmuch as these may provide alternative synthetic reagents, and new possibilities are opened by the existence of these new varieties of halogeno-sugars. Acyl halides interact with the hemi-acetals of the aldehydo-sugars, and 1-chloro, 1-bromo, and 1-iodo-derivatives have been prepared (63).

The characteristic property of galactose to yield furanose derivatives readily is demonstrated in a novel way by the formation in good yield of β -ethylgalactofuranoside when the diethyl- or the dibenzyl-mercaptal of galactose is treated with ethyl alcohol and mercuric chloride at low temperatures in the presence of mercuric oxide (64). Observations in the disaccharide series show that maltose semicarbazone and cellobiose semicarbazone and their acetyl derivatives possess ring structures and, by successive oximation and acetylation of cellobiose hepta-acetate, there is formed a cellobiose-oxime-nona-acetate also containing a ring structure. If, however, cellobioseoxime is acetylated in the cold a nona-acetate of the aldehydo-form is obtained. From the latter, by partial hydrolysis with oxalic acid in methyl alcohol, the corresponding octa-acetate is produced. Finally, by the action of nitrous acid on the octa-acetate there is obtained the octa-acetate of aldehydocellobiose. In this series of changes it is to be noted that the behaviour of the cellobiose derivatives on acetylation is the reverse of that of the corresponding glucose compounds (65).

The benzoyl derivatives of aldehydo sugars have been further investigated. For example, Wolfrom & Christman (66) have treated arabinose-diethylmercaptal with triphenylmethyl chloride and then with benzoyl chloride. To the resulting substance the structure of 5-triphenylmethyl-2,3,4-tribenzoyl-*l*-arabinose-diethylmercaptal is ascribed. Successive removal of the triphenylmethyl group and the mercaptal residues yields a tribenzoyl-*l*-arabinose which probably possesses a pyranose-ring structure. The latter readily benzoylates to the tetrabenzoyl derivative which is obtainable also by direct benzoylation of arabinose. The above tribenzoyl derivative may be obtained also from the tribenzoyl-*l*-arabinosidyl bromide produced by the action of hydrogen bromide in acetic acid on arabinose-tetrabenzoate. Whilst the above reactions are considered to support the pyranose structures of the tri- and tetrabenzoates, the possibility of acyl group migration renders the argument somewhat inconclusive.

A number of benzoates, both open-chain and ring forms, of galactose, mannose, and glucose have also been described.

Knowledge of both natural and synthetic nucleosides has been advanced by several workers. The absorption spectra of adenosine from yeast nucleic acid, and of inosine (obtained by deamination of adenosine) have been compared with those given by methyl derivatives of adenine and hypoxanthine. Gulland & Holiday (67) have shown that the absorption shown by adenosine resembles closely that of 9-methyladenine but differs from that of 7-methyladenine. Again, the absorption of inosine is similar to that of 9-methylhypoxanthine but differs from that of the 7-methylhypoxanthine. It is considered probable from these results that the ribose components in adenosine and inosine are attached at the 9-position in adenine. Inasmuch as muscle adenylic acid gives rise to adenosine under the influence of bone phosphatase, and since the spectra of adenylic acid and 9-methyladenine are almost identical, it is concluded that adenylic acid is probably adenine-9-riboside-5-phosphoric acid. This is a different view from that formerly held, in that the ribose was considered to be attached at the 7-position. An analogy may now be drawn between these nucleosides and flavin in which the sugar residue is attached to the corresponding nitrogen atom (68).

The mode of union of the four mononucleosides in yeast nucleic acid has been the subject of a number of experiments. Guanine-uridylic acid is obtained by gentle hydrolysis and this is found to be monobasic and to contain no free amino group. Union of the guanine and uridylic acid must therefore occur through the phosphate and amino groups; Bredereck & Richter (69) suggest a structural formula which is based on these results.

Experiments on synthetic nucleosides have led to the preparation of substances of the pyranose and furanose series, the latter being of special interest on account of the occurrence of ribofuranose residues in the natural products. Acetobromoglucose and 2,4-diethoxypyrimidine give a condensation product having the glucose residue in position 1. This, on treatment with alcoholic ammonia, yields 1-*d*-glucopyranosidocytosine (70). In the furanose series the synthesis of theophylline-5-methyl-*l*-rhamnofuranoside has been achieved by Levene & Compton (71). This work has involved the preparation and characterisation of both 5-methyl-*l*-rhamnofuranose and 4-methyl-*l*-rhamnopyranose, concerning which there has previously been some confusion. The α - and β -forms of methyl-5-methyl-*l*-rhamnofuranoside are ob-

tained from the two forms of *isopropylidene-l*-rhamnose and give on hydrolysis the free sugar, which shows no observable mutarotation but is not an aldehydo sugar. 4-Methyl-*l*-rhamnopyranose is available from the mixture of 4- and 5-substituted derivatives obtained when *isopropylidenerhamnose* is methylated by the method of Levene & Cortese (72). The acetobromo derivatives of 4- and 5-methyl-*l*-rhamnose combine with silver theophylline in the usual way, and the authors are enabled to compare in detail the behaviour of the theophylline glycosides of 4-methyl-*l*-rhamnopyranose and 5-methyl-*l*-rhamnofuranose.

Another rhamnoside discussed during the period under review is kaempferol-*l*-rhamnoside which is now shown to be obtained by enzymic fission of robinine. In addition, hydrolysis gives rise to a new disaccharide, robinobiose, which is *l*-rhamnosido-*d*-galactose. Since robinine can be hydrolysed, giving a trisaccharide (robinose), it is highly probable therefore that the sequence of monoses in robinose is in the order: rhamnose, galactose, rhamnose (73).

Considerable experimental interest is attached to the synthesis by Freudenberg & Soff (74) of a new disaccharide, 2- β -glucosido- α -glucose. The components brought together in the condensation were 4,6-benzylidene- α -methylglucoside and acetobromoglucose, and by subsequent stages this led to the isolation of hepta-acetyl-2- β -glucosido- α -methylglucoside. The problem of conversion of the methyl-bioside to the free sugar was solved by a choice of reagents which will doubtless find subsequent uses in other similar cases. The hepta-acetyl-methylbioside was brought under controlled conditions into contact with acetic anhydride and sulphuric acid, and subsequent treatment with hydrogen bromide in acetic acid gave the acetobromobiose. The halogen was now replaced by acetyl by means of thallium acetate and the biose octa-acetate was formed. This was de-acetylated to the free biose by the Zemplén method.

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THE CHEMISTRY OF THE LIPINS*

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THE PHOSPHATIDES

It is a known fact that lecithins and cephalins occurring in nature are mixtures of various chemical individuals which differ from each other partly in their fatty acids and partly in the glycerine phosphoric acids (α - and β -glycerine phosphoric acid). Japanese research workers (Suzuki and collaborators) have of recent years made remarkable attempts to separate these mixtures into their single components. Continuing these investigations it was maintained by Yokoyama that it was possible to isolate from the yolk of egg the following lecithins: Dioleo- α -lecithintetrabromide, di-isopalmitic- α -lecithin, oleo-clupanodonic- α -lecithindodecabromide, oleo-isopalmitic- β -lecithindibromide, and dioleo- β -lecithintetrabromide. Likewise, Nishimoto obtained from yolk of egg the cephalins: palmitic-dibromostearic- α -cephalin, palmitic-octabromoarachic- α -cephalin, palmitic-dibromostearic- β -cephalin, palmitic-octabromoarachic- β -cephalin, and di-(octabromoarachic)- β -cephalin. If confirmed, these investigations would seem to furnish the chemistry of phosphatides with methods of surprising promise.

The phosphatides contained in small amounts in milk have been studied more closely by Diemair, Bleyer & Ott as well as by Kurtz, Jamieson & Holm. They comprise, in addition to glycerine phosphatides (lecithin and cephalin), another substance, having the properties of protargon. This confirms the results of earlier investigations by Osborne & Wakeman. By titration with sodium hydroxide it could be established that the purified glycerine phosphatides consisted of 44 per cent of cephalin and 56 per cent of lecithin (Kurtz, Jamieson & Holm). By determination of amino nitrogen in the hydrolysate Diemair, Bleyer & Ott found the same value for cephalin (45 per cent), but a very much lower value for lecithin (23 per cent) on the basis of a choline determination.

The nature of the fatty acids occurring in glycerine phosphatides

* Received February 18, 1937.

has been studied again by various authors. Diemair, Bleyer & Ott found in milk phosphatides palmitic, stearic, and oleic acids. According to Kurtz, Jamieson & Holm, however, palmitic acid is supposed to be entirely absent. They assume the mixture of fatty acids to consist of myristic acid 5.2 per cent, stearic acid 16.1 per cent, arachic acid 1.8 per cent, oleic acid 70.6 per cent, and dicotetrenoic acid (?) 6.3 per cent. Both investigations, however, agree that, contrary to the case of milk fat, there are no lower saturated fatty acids in the glycerophosphatides of milk.

Using a method which has previously been applied for separating the different lecithins of soya bean, Yokoyama & Suzuki isolated from herring roe, as well as from human brain, various α - and β -lecithins and therefrom the following fatty acids: from herring roe—palmitic acid, dibromostearic acid, tetrabromostearic acid, and hexabromostearic acid; from α -lecithins of human brain—oleic acid (determined as dibromostearic acid), arachidonic acid (determined as octabromoarachic acid), and palmitic acid. In addition to the fatty acids already mentioned linoleic acid could be found in the β -lecithins of human brain. The distribution of fatty acids in the bulk of lecithins originating from brain was: (total 100 per cent) oleic acid 85.4 per cent, linoleic acid 1.3 per cent, arachidonic acid 3.3 per cent, and palmitic acid 10 per cent. According to Klenk & Ditt (1) these figures are not entirely reliable. Ault & Brown established that phosphatides of the renal glands of cattle contain particularly large amounts of arachidonic acid (22 per cent of the total of fatty acids). The probability of the presence of more than traces of highly unsaturated fatty acids, besides arachidonic acid, is said to be very small. In addition palmitic acid (24 per cent), stearic acid (11.1 per cent), arachic acid (2.0 per cent), oleic acid (40.2 per cent), and very likely myristic acid (1.2 per cent) were found. Klenk & Dittmer succeeded in finding in glycerine phosphatides from heart muscle, spleen, and renal glands of cattle, not only highly unsaturated C_{20} acids but also highly unsaturated C_{22} acids, present, however, in considerably smaller amounts. Furthermore, Klenk & Ditt (1) determined that the fatty acid mixture of heart-muscle phosphatides from cattle consist of: solid fraction— C_{16} , 14 per cent; C_{18} , 21 per cent; liquid fraction— C_{16} , 5 per cent; C_{18} , 45 per cent; C_{20} , 14 per cent; C_{22} , 1 per cent. Amongst the liquid fatty acids of the C_{18} group linoleic acid and probably also linolenic acid were represented. The C_{20} acids consisted mainly of arachidonic acid but there must have been present, beyond

doubt, some less unsaturated acids of this group. The figure which was found for the group of unsaturated C_{22} acids is probably too low owing to analytical difficulties. As a further contribution to these series of investigations, where a comparison is made of the fatty acids of liver phosphatides with those of liver oils from various species of vertebrates, Klenk (2) has also studied this matter in the case of one reptile (*Testudo graeca*). Thus these investigations have been temporarily ended. A survey of the results obtained thus far shows a remarkable agreement in the composition of the fatty acid mixtures in the liver phosphatides of the different species of vertebrates. Besides C_{16} and C_{18} acids, which commonly occur, there were found, quite regularly, unsaturated C_{20} and C_{22} acids (usually highly unsaturated) in considerable amounts. Liver oils, when consisting principally of triglycerides, contained essentially the same fatty acids as the phosphatides. Quantitatively they differed considerably more than the phosphatides. It is assumed that the triglycerides of liver oils are formed, at least partly, by the esterification of phosphatides.

Irving & Smith report on the fatty acids of the total lipoids of hog liver. The following saturated fatty acids were found: *n*-decanoic acid and lauric acid, 0.4 per cent; myristic acid, 0.7 per cent; palmitic acid, 14 per cent; stearic acid, 18.8 per cent; and arachic acid, 1.7 per cent. Unsaturated fatty acids were present as follows: palmitoleic acid, 1.5 per cent; oleic acid, 28 per cent; linoleic acid, 5 per cent; linolenic acid, absent; C_{20} acids, 20 per cent; C_{22} acids, 7.5 per cent. The paper contains a very thorough and noteworthy criticism of methods of investigation. The $\Delta^{12:13}$ -octadecylenic acid, isolated from hog liver by Hartley (1909), could not be found by Channon, Irving & Smith (1, 2). The main part of the octadecylenic acid present was ordinary oleic acid (85 per cent). The presence of $\Delta^{10:11}$ -octadecylenic acid in small amounts is possible. In the bulk of the lipids from ox blood Parry & Smith found that saturated fatty acids were present as follows: C_{16} , 10 per cent; C_{18} , 13 per cent; higher acids, 3 per cent. Unsaturated acids occurred as follows: C_{18} , 26 per cent; C_{20} , 33 per cent; C_{22} , 10 per cent. Furthermore, the presence of small amounts of lower fatty acids is possible.

Sinclair (1, 2), working with rats, established that the ratio of saturated to unsaturated fatty acids is surprisingly constant when determined on the phosphatides of the total body and when compared with the ratios for single organs such as liver, muscle, kidney, and heart. This constancy prevailed in spite of the fact that marked dif-

ferences existed in the iodine number of total fatty acids which varied both with the quantity and quality of the fats on which the rats were fed. Another paper of Sinclair (3) is concerned with the more precise definition of the exogenous factors which influence the iodine number of phosphatide fatty acids. By feeding the rats on fats containing large amounts of elaidic acid he obtained from liver and muscle phosphatides which were rich in elaidic acid. The absorption of elaidic acid in the liver phosphatides took place rapidly, but proceeded much more slowly in the muscle phosphatides.

Cavanagh & Raper gave rats linseed oil which was saturated with deuterium. After killing the animals, the ether-soluble substances from liver, kidney, and body fat were separated by the acetone method into lipid and glyceride fractions. The amount of deuterium in each fraction was then determined separately. The following figures were found to represent the distribution of deuterium, expressed as percentage of the total hydrogen in the substances: liver lipids, 0.98 per cent; liver glycerides, 0.97 per cent; kidney lipids, 0.63 per cent; adipose tissue, 0.75 per cent. The authors conclude that lipids too are taking part in the total fat metabolism.

Booth & Milroy, as well as Booth and Smyth, obtained from the aqueous extracts of kidney, brain, and liver a substance rich in phosphorus, which yielded choline when split by hydrolysis; it was probably the choline ester of sphingosine phosphoric acid. The substance is, therefore, a partial decomposition product of sphingomyelin. Shortly before, Strack, Neubaur & Geissendörfer (1, 2) had reported on the occurrence of a similar substance in liver and placenta, but they assumed their substance to be a water-soluble sphingomyelin. From ox liver Inukai & Nakahara isolated phosphocholine as a picrate. Phosphocholine may be precipitated with phosphotungstic acid.

Tropp succeeded in finding lignoceryl-sphingosine in cattle lungs, while heart, kidney, lymphatic glands, bull testicles, and white and red bone marrow did not contain this substance.

Kabashima & Suzuki synthesized dipalmitic- β -cephalin and - β -lecithin. The resulting products showed complete agreement in their behaviour when compared with the corresponding natural cephalins and lecithins. Jackson synthesized the amorphous calcium salt of monocholine-phosphoric acid ester by treating choline chloride with phosphoric acid and phosphorus pentoxide, as well as the tricholine-phosphoric ester by treating trimethylamine with tri- β -chlorethylphosphate.

Tait & King observed that the absorption of oxygen by lecithin under aërobic and anaërobic conditions becomes much greater in the presence of glutathione and is much more rapid than that of saturated and unsaturated fatty acids, even of those fatty acids which were obtained by hydrolysis of the investigated lecithin. According to experiments by Page & Bülow, the absorption of oxygen was always greater in the presence of iron salts in the case of cephalin than in the case of lecithin, even if phosphatides having the same iodine number were compared with each other. A comparison between phosphatides and acids of equal iodine number, as regards absorption of oxygen, did not show any difference in their experiments. It seems, therefore, under the prevailing conditions, that the combination of acids resulting in the formation of the phosphatide molecule is of no influence on the rate of oxidation; this is in contrast to the results of Tait & King.

An investigation by Evans shows that vegetable lecithin inhibits autoxidation of vegetable oils caused by the presence of active metals. The reason for this inhibition is at present not known.

Various authors have established a decrease in the content of phosphatides in organs preserved in formaldehyde solution. Epstein & Lorenz showed this decrease to be due to a splitting of glycerine phosphatides caused by the action of acid. King has reported on the action of lecithinase, as found in kidney and intestinal mucous membrane, on divers phosphatides from which the enzyme splits off phosphoric acid. The action was strongest on brominated lecithin, which under suitable conditions could be completely decomposed in the course of twenty-four hours. The action decreased in the following order: bromolecithin, lysolecithin, egg lecithin, liver lecithin, hydrolecithin, cephalin from brain, phosphatide from cabbage leaves, synthetic lecithin, di-stearo-phosphate. The action was independent of pH in the last two substances. According to Rossi, an enzyme is found in the milt, but is present with less activity in many other organs also, which, similar to the one previously mentioned, separates phosphoric acid from the so-called polydiaminophosphatide (sphingomyelin). An enzyme prepared from hog kidney is able to split lecithin but not polydiaminophosphatide. The activity is increased by magnesium ions and salts of bile acids and is diminished by phosphates and arsenates. Francioli (2) established the existence of an enzyme in the fungus *Lycoperdon giganteum* which decomposes lecithin and lysolecithin within the range, pH 7.0 to 5.5. The products of decomposition are

choline, fatty acids, and phosphoric acid. As to whether the lipase properties of the fungus extract, which were also observed, were due to the same enzyme or to a different one could not be settled.

Using an enzymatic method Rae found that glycerophosphoric acid consists mainly of the β -acid when originating from egg yolk, and of equal parts of a mixture of α - and β -acid when prepared from liver lecithin; it consisted mainly of the α -acid when prepared from brain lecithin, brain cephalin, and phosphatidic acid. In some cases of constitutional hemolytic anemia, and splenomegalic liver cirrhosis accompanying hemolytic icterus and pernicious anemia, Kanócz found a lecithinase in the serum. Francioli (1) observed that lecithin gradually disappeared and lysolecithin appeared when powders of various organs, which had been prepared by drying the organs *in vacuo*, were kept for some time. This is assumed to be due to enzyme action (lecithinase A).

PHYSICAL CHEMISTRY OF PHOSPHATIDES

Bull & Frampton determined the isoelectric point of mixtures of egg lecithin and egg cephalin. It was found to be a function of the amino nitrogen content of the mixture. By extrapolation of their experimental results it could be established that lecithin has its isoelectric point at pH 6.4. In agreement with the results of Sueyoshi aqueous solutions of lecithins showed a shifting of the isoelectric point on ageing. Chain & Kemp made a cataphoretic determination of the isoelectric points of lecithin and sphingomyelin. The value for lecithin was $\text{pH } 6.7 \pm 0.2$, as compared with a theoretical value of pH 7.5, computed from the dielectric constant. The discrepancy is attributed to a rapid spontaneous decomposition of the lecithin, giving free fatty acids [Fischgold & Chain (2)]. The isoelectric point of sphingomyelin, by reason of its structure, should agree with that of lecithin. A more accurate determination was rendered doubtful because it was impossible to prepare an absolutely pure sphingomyelin. The specimens were accompanied, persistently, by a substance of acid nature which could be only incompletely removed. However, it may be concluded from measurements carried out on systems of increasing purity that pure sphingomyelin possesses an isoelectric point of pH 6.

Price recorded the spontaneous decomposition of lecithin under exclusion of air, as observed by Fischgold & Chain (2), by investi-

gating the change, with ageing, in the electrophoretic properties of lecithin sols. He found that the electrophoretic velocity of a lecithin sol at constant pH (5.02) and constant temperature (20° C.) was 15 cm./sec. 10^6 and increased in spite of exclusion of air. At first the increase in velocity was linear; later it proceeded more slowly, and in the course of forty days attained a value of 90 cm./sec. 10^6 ; it remained constant from then on for about five months. Furthermore, he observed an increase in the isoelectric point with increasing age of lecithin, the cause of which was believed to be the adsorption of free fatty acid ions on lecithin micelles.

Kuhn, Hausser & Brydówna investigated the dielectric properties of phosphatides. Hausser, Kuhn & Giral have carried out measurements of the dielectric constant as a function of concentration, temperature, and wave length, in alcoholic solutions of sphingomyelin, using a resonance circuit. They observed a strong anomalous dispersion which was independent of viscosity and must, therefore, be ascribed to a novel molecular resonator of an extraordinarily low characteristic frequency. The strongly polar group of the sphingomyelin molecule swings like a pendulum under the action of the high frequency field and under the influence of the directing force of the long resting arms formed by the two weakly polar chains.

Fischgold & Chain (2) developed a method for determination of the acid- and base-binding capacity of various phosphatides. According to these authors all phosphatides in acid solution can take up one equivalent of hydrogen ions, whereas in alkaline solution only those phosphatides possessing an amino group can give off one equivalent of hydrogen ions. This is in favour of the assumption that lecithin, lysolecithin, and sphingomyelin are present only as cations or zwitterions, cephalin and lysolecithin as neutral molecules, anions, or cations. Similar results were obtained by Jukes, who titrated, electrometrically, mixtures of lecithin and cephalin in 98 per cent alcohol. Spiegel-Adolf (3), also, reports on the acid-base binding capacity of cephalin.

General directions for obtaining clear phosphatide sols from trade preparations of phosphatides are given by Jong, Verberg & Westerkamp. Jong & Saubert report on complex flocculation or coacervation, respectively, of lecithin sols caused by simultaneously adding sodium arabinat and lanthanum nitrate; the difference from types previously known rests in the complex relationship between the three interacting components: colloidal zwitterion, colloidal anion, and crys-

talloidal cation. They act in such a way that the crystalloidal cation is in relation with the negative end and the colloidal anion is in relation with the positive end of the zwitterion. Jong & Saubert also succeeded in attaining an analogous complex flocculation involving colloidal zwitterion, crystalloidal cation, and crystalloidal anion, by adding simultaneously calcium chloride and ammonium molybdate to lecithin sols.

In order to explain the rôle of lecithin in the resorption of fats, Fürth, Breuer & Herrmann investigated the influence of lecithin on the diffusion of the fatty acid-bile acid complex into gels and through membranes. They found that diffusion increased or was inhibited according to the nature of the gel or membrane.

According to Bamberger (1, 2) lecithin brings about an increase in the swelling capacity of gelatin gels by from 12 to 15 per cent independently of the pH. The amount of bound water, however, is diminished. Also, lecithin has a measurable influence on the elasticity of gelatin gels. The elastic modulus of twisting and shearing and resistance to fatigue are diminished, and capability of plastic deformation is increased. Cholesterol quite generally shows the opposite behaviour, being an antagonist to lecithin in these instances also. The author points out that the fluctuations in swelling power, as observed in living cells, cannot be caused alone by shift of hydrogen-ion concentration or salt effect, but that cell lipoids must play a certain part. Furthermore, mention is made of the biological importance of these results for the problem of muscular contraction. When swelling of gelatin takes place in a salt solution where the ratio of Na:Ca:K is 10:1:1 the amount of swelling is slightly decreased upon addition of lecithin, but it is strongly increased upon addition of cholesterol (Moraczewski & Sadowski). Przyłeczki & Majmin (2) observed a marked increase in the adsorption of glycogen and dextrin on the fatty surface of olive-oil emulsions when a small amount of lecithin was added to the olive oil. Spiegel-Adolf (2) reports on the action of cephalin from human brain as a protective colloid on gold sols, serum albumin, serum globulin, lecithin, and cholesterol. She also reports (1) on the relationship between the iodine number of monophosphatides and their behaviour toward neutral salts, especially bromides. According to previous studies it appeared that discrepancies existed in respect to the position of bromides in the Hofmeister series and their influence on viscosity and refractivity of egg-lecithin

sols. Spiegel-Adolf now shows that a parallelism exists between iodine number and the specific action of bromides on egg-lecithin sols as well as on sols of human lecithin and cephalin.

By means of surface-potential measurements according to the method of Schulman and Rideal, Hughes (1) investigated the physical and chemical properties of monomolecular films of lecithin, lysolecithin, cholesterol, tripalmitin, and triolein. He calculated, from the variation in surface potential of the substances mentioned, the relative electric moments of their molecules. In contrast to cholesterol, tripalmitin, and triolein, lecithin and lysolecithin showed a variation of their physical and chemical behaviour in the range of pH 3 to 6 (lecithin) and 2 to 5 (lysolecithin), as a consequence of ion formation. Using the same method Hughes (2), furthermore, investigated the effect of lecithinase obtained from various snake venoms by permitting them to act on monomolecular lecithin films and following the simultaneous change in surface potential. As a result the degree of hydrolysis of the lecithin was found to depend on the pH, the surface concentration of lecithin molecules, and the venom concentration. The optimum pH was 7.3. Condensation of lecithin molecules resulted in decrease of hydrolysis. Lecithinase proved to be stable toward prolonged boiling at a pH of 5.9, but became rapidly inactive when boiled at a pH greater than 7.0.

Schmitt, Bear & Clark, in the x-ray analysis of dried nerve fibres, obtained diagrams showing meridian periods of 4.2, 4.7, 5.2, and 5.9 Å. Since these rings corresponded to the extracted and dried lipoids, lecithin (4.2 and 4.7 Å.) and cholesterol (5.2 and 5.9 Å.), respectively, and since different diagrams were obtained when fresh nerve fibres were x-rayed the authors assume that drying of nerve fibres in the destruction of lipoids of the myelin sheath.

CEREBROSIDES

Much has been said about the chemical constitution of the cerebronic acid that is present in cerebrin, which Klenk assumes to be an α -hydroxy lignoceric acid. In various studies Levene and collaborators have tried to prove that cerebronic acid consists of a mixture of different hydroxy acids [Taylor & Levene; Levene & Heymann; Levene & Yang (1)]. Their basis is the fact that they obtain as oxidation products of cerebronic acid, even after protracted purification

of the material, fatty acids having 22, 23, and 24 carbon atoms. In contrast to this, Klenk (3) was able to reestablish his previous findings according to which the main product of oxidation of cerebronic acid is tricosanic acid. In addition, however, the oxidized mixture contained lower fatty acids which, according to experiments on the oxidation of α -hydroxy stearic acid, are always present in such cases as by-products. Levene & Yang (2) report that they did not succeed in obtaining lower fatty acids as products of oxidation when oxidizing α -hydroxy stearic acid. However, Klenk & Ditt (2) obtained from α -hydroxy stearic acid, in analogy to those substances formed as products of oxidation of cerebronic acid, not only margarinic acid but more than 20 per cent of lower fatty acids.

In a paper, likewise on the constitution of the fatty acid part of phrenosin (cerebron) and kersin, Chibnall, Piper & Williams, principally by x-ray analysis, found that the products of oxidation of cerebron consist of 85 per cent of $C_{23}H_{46}O_2$ and 15 per cent of $C_{25}H_{50}O_2$. They conclude that their phrenosinic acid is a mixture of 85 per cent of $C_{24}H_{48}O_3$ and 15 per cent of $C_{26}H_{52}O_3$. A similar result was reached by Crowfoot, who investigated by x-rays two specimens, namely, cerebronic acid proper and oxidised cerebronic acid, both supplied to her by Klenk. According to her investigations the cerebronic acid as prepared by Klenk is of a somewhat higher purity than were the specimens investigated by Chibnall and collaborators.

That the problem of the constitution of cerebronic acid is not likely to be solved by the method of the melting point or mixed melting point is evident from a paper by Ashton, Robinson & Smith. They investigated the melting diagram of mixtures of tricosanic and tetracosanic acid. They found that these acids form a continuous series of solid having one melting point minimum. Mixtures having a content of tetracosanic acid of from 0 to 40 per cent showed a difference in melting point of only $0.5^\circ C$.

For the first time Abderhalden & Schwab have carried out successful experiments on the enzymatic splitting of cerebroside. They showed that the amide bond between fatty acid and sphingosin in cerebron is split by trypsin. The trypsin sample used was free from erepsin. Nevertheless, cerebroside is in some cases only slightly digestible. For instance, Beumer & Fasold, after the oral administration of 1 gm. of cerebroside to an infant, could recover 0.9 gm. in the faeces. From the faeces of a boy it was possible to recover a

great deal of administered cerebroside, whereas in another case the amount recoverable was considerably less. An excretion of sphingosine in urine after the feeding or intravenous injection of cerebroside into rabbits and dogs, which was reported to have been observed by others, could not be confirmed. Injection experiments with rabbits and dogs showed that cerebroside may be stored by various organs and remain there intact for quite a long period. Kimmelstiel & Laas obtained a similar result. Repeated intravenous injection into rabbits of cerebroside emulsions produced a storage of cerebroside in spleen and liver of a type similar to that of Gaucher's disease. Gradually the cerebroside disappeared from the reticular cells, which seems to indicate that the substance was slowly decomposed. An intermediate decomposition product, psychosin (galactosido-sphingosin), is decomposed by emulsin, as could be shown by Helferich, Appel & Gootz from galactose formation.

Thannhauser & Reichel were able to show, in agreement with previous work by Rosenheim, that pancreatic lipase and emulsin alone are not able to decompose cerebrin. A marked decomposition takes place, however, if activators such as hydrogen sulphide, cysteine, reduced glutathione, or *l*-ascorbic acid are added to the system. The activating property of cysteine can be neutralised by iodoacetic acid. Furthermore, the authors established that the activators of cerebroside have an inhibiting influence on the activity of polydiaminophosphatase.

The question raised some years ago by Trier as to whether cerebroside-like substances are present in rice is answered in the negative by Arni.

Tropp & Eckardt found the dried residue of a cyst, probably originating from trauma, to consist of 72 per cent of cerebroside, mainly cerebrin and kersin; no phosphatides were present. The authors propose that the name "cerebrosideomes" should be used for such cerebroside-containing cysts.

VEGETABLE AND BACTERIAL PHOSPHATIDES

According to Channon & Foster the phosphatides of wheat germ consist of phosphatidic acid, lecithin, and cephalin in the approximate proportion of 4:4:1, respectively. In order to prepare from the germ an extract rich in phosphatides but containing the least possible

amount of non-phosphatidic phosphorus it has been found especially desirable to use as an extracting solvent a mixture of equal parts of alcohol and ether. In the fraction containing lecithin and cephalin the presence of choline and colamine could be established by isolating the corresponding gold salts. The fatty acids of these wheat-germ phosphatides have been studied more closely by Diemair & Bleyer. Of the saturated fatty acids palmitic acid only was found. The greater part of the unsaturated acids was represented by linoleic acid. The phosphatide fraction obtained by Diemair & Bleyer showed the presence of a small amount of sugar (2.35 per cent, calculated as glucose, a figure which is in agreement with the 3 per cent found for the lecithin-cephalin fraction by Channon & Foster). According to Javillier & Colin the amount of phosphatide phosphorus, expressed in terms of total phosphorus, is 9 per cent in the case of wheat seedlings and 12 per cent in lentil seeds. According to Nottbohm & Mayer the phosphatides contained in wheat flour may be extracted much more efficiently with benzene-alcohol when the flour is moistened. Arni was able to split a crude phosphatide obtained from wheat or wheat gluten, respectively, into lecithin free from sugar and into a sugar-containing constituent with 1.06 per cent phosphorus. Confirming previous results obtained by Ivata he succeeded in isolating a (palmitic-) lysolecithin from rice. He obtained phosphatidic acid from castor oil and nettle. In crude phosphatides from rhubarb leaves, choline and glycerophosphoric acid could be demonstrated. Green leaves from divers plants showed, according to Echevin, a content of phosphatide phosphorus of from 20 to 54 mg. per cent of the dried substance. With leaves which had turned yellow in autumn the corresponding values were from 0 to 3 mg. per cent only. Bengis & Anderson obtained from coffee-bean fat very small amounts of phosphatides (0.1 per cent) having a content of 3.84 per cent phosphorus and 0.89 per cent nitrogen. Romoli-Venturi & Pugliese report on the chemical structure and biological properties of the lipid complexes of *Daucus carota*. Sastri & Sreenivasaya have investigated the lipoids occurring in the seeds of fenugreek (*Trigonella foenum graecum*). By use of a method of separation which they describe, they report the occurrence of a phospholipoid linked with sphingomyelin; lecithin, a galactolipoid soluble in pyridine, and a galactoside as well as a sulphur-containing galactolipoid insoluble in pyridine were also found. However, closer investigation of the substances discovered is still lacking.

According to Crowder & Anderson the total lipoids of *Lactobacillus acidophilus* consist of about one-third of a phosphatide with phosphorus and nitrogen contents lower than is to be found in vegetable and animal phosphatides (phosphorus, 1.45 and 1.40 per cent, respectively, and nitrogen 1.21 per cent). Hydrolytic decomposition yielded: 55 per cent fatty acids, 20 per cent of a crystalline polysaccharide and small amounts of glycerophosphoric acid and choline. The fatty acid fraction was essentially a mixture of palmitic acid, stearic acid, a saturated higher fatty acid (probably tetracosanic acid) and unsaturated C_{16} and C_{18} acids. The polysaccharide obtained by decomposition with alkali was decomposed by further hydrolysis with acid, yielding *d*-galactose and apparently fructose and glucose. An appreciable amount of phosphoric acid originally present in the phosphatide does not occur as glycerophosphoric acid but is linked to the polysaccharide. One fraction of polysaccharide with the high phosphorus content of 9.5 per cent was isolated. The choline nitrogen amounted to only a very small proportion of the total nitrogen. The nature of the other nitrogen-containing substances is as yet unknown. Attempts to isolate colamine were unsuccessful.

Macheboeuf, Lévy & Fethke (1, 2) tried to isolate from the lipoids of the tubercle bacillus single substances which could be chemically well characterised. It has not yet been settled as to whether their attempts have been successful. They obtained a phosphatide, distinguished by a hapten effect, having 4.9 per cent phosphorus and 1.9 per cent nitrogen, yielding 67.0 per cent fatty acids and 6.25 per cent sugar (calculated as galactose), but no glycerol. Another substance, chemically very similar to the one just mentioned, was obtained by Macheboeuf & Cassagne from the diphtheria bacillus. This substance, too, showed the hapten effect. Other substances isolated by Macheboeuf and collaborators from tubercle bacilli showed a surprisingly low phosphorus content (0.66 per cent and 0.77 per cent) and contained sugar. Of the carbohydrates set free by acid hydrolysis, a pentose is said to be present. In subsequent papers which report on the isolation of the lipid-like substance with hapten properties, Macheboeuf and collaborators conclude that it is free from nitrogen but contains a large amount of phosphorus.

Hecht (1, 2) believes that he has established the presence of lecithins and stearins in human and bovine tubercle bacilli. He was able to obtain, after hydrolysis of the tubercle bacillus phosphatides with *N*/10 hydrochloric acid, a chloroplatinate which after decomposition

could be identified by biological methods as a choline salt. The substances found by him could not be investigated more closely for lack of material.

Two papers by Bloch deal with a close study of a lipid from human tubercle bacilli, described some time before by Anderson as A3-phosphatide. In these papers Bloch was able to show that the presence of lecithin, cephalin, or sphingomyelin in A3-phosphatide can be excluded with certainty. According to Dessy & Francioli "phosphatides in the common meaning of the word" are present in tubercle bacilli only in very small amounts.

Strong & Peterson obtained, by precipitation with magnesium chloride from the acetone solution of the lipid mixture of *Aspergillus sydowi*, a phosphatide containing phosphorus, nitrogen, and magnesium in the proportions 1:2:2, respectively. After removing magnesium, by shaking with dilute hydrochloric acid, an acetone-soluble phosphatide was obtained. According to Hettche phosphatides are also found in lipid extracts of *pyocyaneus*, but no details are given.

Salisbury & Anderson isolated lecithin and cephalin from beer yeast (*Saccharomyces cerevisiae*). Saponification yielded two solid fatty acids, namely, palmitic acid and stearic acid. The bulk, however, was represented by liquid fatty acids (84 to 86 per cent), the catalytic hydrogenation of which resulted in a mixture of stearic and palmitic acids.

DISTRIBUTION OF PHOSPHATIDES IN TISSUES, ORGANS, AND BODY FLUIDS

By means of a method proposed by Karrer for the determination of α - and β -glycerophosphoric acid the distribution of α - and β -lecithin was determined in the brain of adults, new-born infants, and foetus by Antoniani. In all three cases the β -form predominated.

Kuriyagawa and collaborators find that there is no appreciable difference in the content of phosphatides or other lipoids present in the plasma and corpuscles from blood of the left and right ventricle of the heart, either normally or during postprandial hyperglycemia. Therefore, it seems that the lungs take no specific part in the metabolism of fats and lipoids. Boyd (2) observed only small changes in the phosphatide content of human blood plasma during the course of the day. Page, Kirk, Lewis, Thompson & Van Slyke investigated,

by means of the gasometric method described by Kirk, Page & Van Slyke, the lipoids of plasma from normal healthy men of different ages. They did not find, between the age limits of twenty and ninety years, any noteworthy variation, either in amount or in composition, of plasma lipoids. The average for phospholipoids, calculated for sixty-six men, was 181 mg. per cent. They also compared critically different methods of analysis and commented on statements made by different authors on the plasma lipoids of normal men. According to Chaikoff & Kaplan, depancreatized dogs when treated with insulin show a decrease of almost all blood-lipoid constituents. In particular, the phosphatide content in the preprandial state was diminished. Brockett, Spiers & Himwich found, after feeding the animals on fat, a small increase of phosphatides in the thoracic lymph. Süllmann & Wilbrandt observed a very marked increase—up to five times the original amount—in the intestinal lymph. Gautenberg established that in cases of pernicious anemia and secondary anemia the phosphatide contents in the blood serum fluctuate rather erratically. In two cases of marked lipemia due to diabetes Herbert found in the plasma 0.74 (0.57), and 0.95 per cent of phosphatides; the phosphatide content of the plasma fat was 7.7 (6.21), and 4.1 per cent, respectively.

Aylward, Channon & Wilkinson found that the phosphatide content of the livers of rats, on diets rich in fat and free from choline, decreases up to the seventh hour from 3.51 per cent to 2.65 per cent but is restored almost to the original value after thirteen hours. Addition of choline chloride prevents this marked decrease in the phosphatide content. According to Chaikoff & Kaplan the degenerated fatty livers of depancreatized dogs contain only 1.02 to 1.08 per cent of phosphatides as compared with 1.74 to 2.20 per cent in normal cases. The phosphatide content in liver is not appreciably altered by injecting insulin, according to Abrami *et al.* Cyclical variations in the phosphatide content during the course of the day could not be observed by Ohlsson & Blix, whereas such variations were demonstrated in neutral fat of the liver. Breusch & Scalabrino determined the amount of phosphatides and other lipoids of the liver in various diseases and found that the values for phosphatides are lower than normal in cases of hypertonia and liver cirrhosis. New-born infants also show low values because of their high water content.

A considerable increase in phosphatides was observed by Pasternak & Page (1) regularly in the skeletal muscles of rabbits and rats after doses of tyrosine and thyreoidin had been given, but no such

increase was found in the heart muscle, nor were appreciable changes observed in brain. Liver and blood showed fluctuations in the phosphatide values. According to preliminary experiments with rats, 3,5-bromo-iodotyrosine seems to be an antagonist to thyreoidin as regards its action on the phosphatide content of muscle. In completion of previous researches Pasternak & Page (2) were able to show also that phosphatides, as introduced into the body, are not stored, but are completely used. An observation made previously that thyreoidin causes an increase of almost 100 per cent in the phosphatide content of rat muscle was amplified by stating that the increase cannot be explained by migration from other tissues but must be assumed to be absolute. The authors say that an increased formation of phosphatides probably occurs, brought about by the action of thyreoidin in the organism; these phosphatides are possibly formed as intermediate products in the decomposition of fats. Schmidt, too, observed an increase in the phosphatide content of skeletal muscle. He analysed the fatty acids of phosphatides and non-phosphatides in blood, liver, and skeletal muscle. However, Schmidt & Bradford report that the phosphatide content of muscle remains constant after injection of thyroxine.

A number of papers is concerned with the relationship between phosphatide content of body cells and the degree of their activity. Bloor & Snider showed the phosphatide content of muscle to be dependent on its functional state, those muscles used to the greatest extent showing the highest phosphatide content. An analysis of lipoids of the jelly of Wharton in the human umbilical cord showed, according to Boyd (3), the following mean values (expressed in mg. per 100 gm. of wet substance): total fatty acids, 131; lipoids, 209; neutral fat, 54; phospholipoids, 120; total cholesterol, 33; cholesterol esters, 3; free cholesterol, 31. Although it appears that the value for total lipoids and, in particular, for phospholipoids is relatively small, the low content of cholesterol esters leads to the conclusion, according to Boyd, that one has here to do with inactive, though not with degenerated, tissue. The nature of the relationship between activity and phospholipoid content in active cells becomes clear from another paper by Boyd (6), in which it is demonstrated that active leucocytes also have a higher phosphatide content than inactive ones. He is satisfied that, within certain limits, the phospholipoid content of leucocytes has a valuable prognostic significance in cases of grave infections and like diseases; the greater the phos-

pholipoid content, the better are the chances of recovery supposed to be. The phospholipoid content in leucocytes of normal men, according to Boyd, amounts to 844 mg. per cent with fluctuations of about 241 mg. (average in twenty-two normal persons).

Boyd (1) finds the phosphatide content of rabbit ovaries during pregnancy to increase up to the fourteenth or sixteenth day; the maximum increase is 300 per cent. In the last half of pregnancy the original value is again reached. Parallel with the decrease in phosphatides goes an increase in neutral fat. In guinea pigs, however, the same author (4) could not find any variation in the content of phospholipoids during pregnancy. Here, the phospholipoid content within and without the pregnancy period averaged 1250 mg. per cent (810 to 1870 mg. per cent). From this, and from the fact that it is possible to remove the ovaries of pregnant animals after the first half of pregnancy without interrupting the latter, Boyd concludes that in guinea pigs there is no increase in physiological activity of the ovaries during pregnancy. On the other hand, again according to Boyd (5), the phospholipoid content of the placenta of guinea pigs increases from between the twentieth and fortieth day of pregnancy. The increase continues until the end of pregnancy. This seems to signify an increase in function of the placenta, connected either with the transfer of lipoids from the mother to the foetus or with other physiological functions, e.g., with the production of sex hormones.

Roepke & Hughes report that the amounts of total phosphorus and lipid phosphorus in the blood serum of laying hens are three times the amounts found in the serum of non-laying hens, the inorganic phosphorus content being only slightly changed. Kugler reports his results of a systematic investigation of the content of ether-soluble phosphorus, lecithin, and cephalin in embryos and yolk sac of chickens during development. An inverse relationship is observed between the decline in the content of lecithin and cephalin in the yolk sac and the increase in embryonic tissue. The lipid metabolism attains a climax between the fifteenth and the seventeenth day of development.

Bierich & Lang report on the phosphatide content of malignant ulcers. According to Yasuda & Bloor, carcinomas of men and of mice contain a greater amount of fatty substances, especially phospholipoids, than fibrosarcomas, neurofibromas, fibromyomas, and colloid adenomas of thyreoidea. The higher this content the more malignant the tumor seems to be. In an investigation of the blood of chickens suffering from sarcoma, Pentimalli & Schmidt found an

increase in the total phosphatides. The lipid phosphorus, as determined by Whitehorn's method, was found to average 24 per cent higher than normal.

In a case of Niemann-Pick disease Klenk (1, 4) isolated sphingomyelin from spleen, liver, and brain. The preparations from spleen and liver were mixtures of palmito-, stearo-, lignocero-, and nervo-sphingomyelin, whereas the preparation from brain was practically pure stearo-sphingomyelin. The sphingomyelin represented, especially in spleen and liver, the main portion of the total phosphatides. The high phosphatide content in these organs was shown to be due to a large storage of this particular phosphatide group. The content of glycerophosphatides corresponded approximately to that of normal organs. Furthermore, it could be shown that the glycerophosphatides of liver and brain differed in their content of highly unsaturated C_{20} and C_{22} acids, a fact which is probably connected with the different content of C_{24} acids in the sphingomyelins of both organs. Only traces of cerebroside were present in Niemann-Pick brain whereas a sugar-containing substance could be isolated. Epstein was not able to establish an increased content of phosphatides in a case of infantile amaurotic idiocy (Tay-Sachs), contrary to what is found in the brain in Niemann-Pick's disease. He concludes that the disturbances in metabolism responsible for these two diseases are different. In two cases of Schüller-Christian disease Cowie & Magee found the phosphatide content to be normal in all organs examined, namely, 2.24 and 1.97 per cent in liver, and 1.53 and 1.43 per cent, respectively, in spleen, calculated from the fresh tissue weight.

Waddington *et al.* state that the evocator substance, described by them previously, which promotes the development of amphibian ectoderm under certain conditions in growth experiments *in vitro*, is not cephalin. Mayer, investigating the effect of lipoids on the growth of tissues *in vitro*, made use of a lipoid described by Fischer & Hecht, which, according to them, was obtained from hog brain and consists of a special cephalin soluble in alcohol and cerebroside. It transpired that the usually observed growth-promoting action of embryonic extracts on tissue cultures was diminished or stopped altogether by the lipoid emulsions.

Cephalin from cattle blood or cattle brain enhances, while lecithin and cholesterol inhibit the clotting of rabbit blood (Okamura).

METHODS OF QUANTITATIVE DETERMINATION

Katsura *et al.* (1) have worked out a method for the simultaneous determination of phosphatides, cholesterol, esterified cholesterol, and neutral fat in blood; the method requires a sample of 0.1 cc. only; the phosphatide content is computed by a difference method. Some suggestions for improvement are made in a later publication by the same authors (2). Kirk, Page & Van Slyke (2) describe a method for the determination of total lipoids, free cholesterol, lipid amino nitrogen, and total phosphatides in blood plasma, blood corpuscles, and tissues. The method requires 3 cc. of plasma, 3 to 3.5 cc. of blood corpuscles, or 300 mg. of tissues. The content of phosphatides is calculated from the amount of phosphorus contained in the total lipoids. In this case the determination of phosphorus is carried out by the method of Kirk. Stewart & Hendry show how the phosphatide content of blood may be computed with sufficient accuracy from the phosphorus content of the ether-alcohol extract of blood. They found, in a specimen of phosphatides prepared from blood, that the ratio of fatty acids to phosphorus was 1.5:1 and believe themselves justified in concluding that the blood phosphatides consist of equal parts of sphingomyelin and glycerophosphatides (lecithin and cephalin). According to Turner, the phosphatides and cholesterol of serum are precipitated to the amount of 95 per cent, together with the protein, when the protein-precipitation method of Folin & Wu is used. Therefore, the precipitate may be used for the determination of phosphatides in case the amount of available serum is insufficient. For the determination of lecithin, i.e., phosphatide phosphorus, in watery foodstuffs, Grossfeld & Walter recommend isopropyl alcohol as an extracting agent, or, alternatively, in the presence of large quantities of fat, a mixture of isopropyl alcohol and benzene (5:4 parts by volume respectively). In modifying a method described by Green for the colorimetric determination of phosphorus in blood serum by means of Denigès reagent, Roepke recommends the incineration of organic material in the presence of magnesium nitrate. This paper contains instructions in regard to the determination of total phosphorus, lipid phosphorus, and the total acid-soluble phosphorus of serum. From the variations in the amounts of lipoids, phospholipoids, and cholesterol in ether extracts of blood serum, following upon the addition of different amounts of alcohol to the extracting agent, Grigaut draws conclusions with respect to the decomposition of lipoproteins. The

paper mainly discusses the conditions under which the extraction of lipoids and cholesterol from blood serum by means of alcohol-ether mixtures can be best effected.

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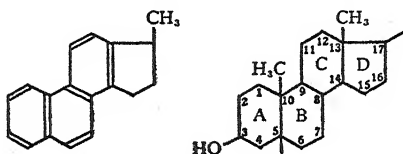
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The term steroid has been proposed by Callow & Young (1) for those compounds which are chemically related to cholesterol and contain a hydrogenated cyclopentenophenanthrene-ring system, including therefore the sterols proper, the bile acids, cardiac aglycones, toad poisons, saponins, and sex hormones. The chemistry of the sterols and bile acids was last reviewed in these reports (2) in 1933, at a time immediately after the revision of the old formulae. The new formulae not only permitted a reformulation of the older facts but also offered a more precise approach to synthetic manipulation of the steroid molecule. In the intervening three years the stimulus thus given to steroid research has resulted in an enormous quantity of important work. The field has been recently reviewed in the excellent and thorough monographs of Fieser (3) and of Lettré & Inhoffen (4). The physiology of the bile acids has been reviewed in the monograph of Shimizu (5), and shorter reviews, by Elderfield (6) on the cardiac aglycones, by Sobotka (7) on the chemistry of bile acids and sterols and by Bills (8) on the physiology of the sterols, have also appeared. It is impossible in the available space to include all of the important developments of the last three years. In all cases we have preferred to discuss those compounds and reactions possessing physiological implications, but even with this limitation considerable omission and rigid condensation have been necessary.

Dehydrogenation of steroids.—The steroids as a group are uniquely characterized by their dehydrogenation with selenium or palladium to certain aromatic hydrocarbons in which the original cyclic carbon skeleton is intact. The reaction serves as an ultimate test for the reduced cyclopentenophenanthrene nucleus and for the inclusion of a compound in the steroid group. Cholesterol, depending upon the conditions, yields either chrysene or the Diels hydrocarbon, $C_{18}H_{16}$ (9, 10, 11, 12, 13). Synthesis of the latter compound by Harper, Kon & F. C. J. Ruzicka (14) has definitely established it as γ -methyl cyclopentenophenanthrene (I). Selenium dehydrogenation of the toad

* Received January 5, 1937.

I. γ -Methyl cyclopentenophenanthrene

II

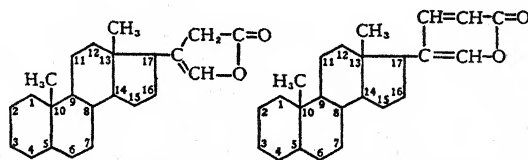
TABLE I
CARDIAC AGLYCONES

Genin	Formula	M.P.	Hydroxyls at Carbons	Relation of C_3 Hydroxyl to C_{10} Methyl	Fusion of Rings A and B
Digitoxigenin	$C_{23}H_{34}O_4$	250°	3, 14	<i>trans</i>	<i>cis</i>
Gitoxigenin	$C_{23}H_{34}O_5$	235°	3, 14, 16	<i>trans</i>	<i>cis</i>
Digoxigenin (147)	$C_{23}H_{34}O_5$	222°	3, 11, 14	<i>trans</i>	<i>cis</i> §
Strophanthidin (147)	$C_{23}H_{32}O_6$	175°	3, 5, 14 CHO at 10	<i>trans</i>	<i>cis</i>
α -Anhydro-uzarigenin (146)	$C_{23}H_{32}O_3$	265°	3, 14	<i>cis</i>	<i>trans</i>
Periplogenin	$C_{28}H_{34}O_5$	185°	3, 5, 14	<i>trans</i>	<i>cis</i>
Sarmentogenin (41)	$C_{28}H_{34}O_5$	266°	3, 11, 14	<i>trans</i>	<i>cis</i> ¶
(Ouabagenin) (141)	$(C_{23}H_{34}O_8)^*$		3, 5, 7, 11, 14 CH_2OH at C_{10}	<i>trans</i>	
(Convallagenin) (142)	$(C_{23}H_{32}O_8)^\dagger$		3, 5, 8, 14	<i>trans</i>	
(Thevetogenin) (143, 144)	$(C_{23}H_{34}O_4)^*$		3, 14	<i>cis</i>	
Scillaridin-A	$C_{24}H_{30}O_8^\ddagger$	250°	14		

* Genin not isolated.

† Genin not isolated; double bond, 9:11 (141).

‡ Genin not isolated; double bonds, 5:6, 7:8 (Stoll).

§ *Trans* fusion of rings B and C.¶ *Cis* fusion of rings B and C.Nucleus of aglycones
except Scillaridin-A

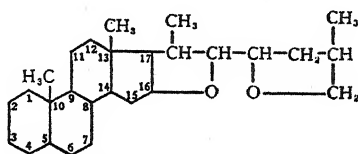
Nucleus of Scillaridin-A

poisons, bufotalin (15) and cinobufagin (16, 17); of the cardiac aglycones, strophanthidin (18) and anhydro-uzarigenin (19); of the

TABLE II

SAPOGENINS

Genin	Formula	M.P.	Hydroxyls at Carbons	Relation of C ₃ Hydroxyl to C ₁₀ Methyl	Fusion of Rings A and B
Digitogenin (160)	C ₂₇ H ₄₄ O ₅	253°	2, 3, 6		
Gitogenin (160)	C ₂₇ H ₄₄ O ₄	273°	2, 3		<i>trans</i>
Tigogenin (159)	C ₂₇ H ₄₄ O ₃	204°	3	<i>cis</i>	<i>trans</i>
Sarsasapogenin (161)	C ₂₇ H ₄₄ O ₃	183°	11		



Nucleus of sapogenins

saponins, sarsasapogenin (20) and gitogenin (21); and of ergosterol (22) and lumisterol (23) also yields the same hydrocarbons. Failure to obtain the hydrocarbons from tachysterol (24, 25), vitamin D (24, 25), isocholesterol [a mixture of agnosterol and lanosterol (26)], and certain of the saponins [which give sapotalene (27)], indicates their nonsteroid nature and the absence of the hydrogenated cyclopentenophenanthrene nucleus.

Interconversions of the steroids.—The production of γ -methyl cyclopentenophenanthrene is final proof of the steroid nature of a substance, but the reaction requires considerable amounts of material for its application. The steroid relationship has been frequently established, therefore, by degradation or transformation of an unknown compound to a steroid of known structure. The first example of this method of attack was the demonstration of the chemical relationship between cholesterol and the bile acids by the conversion of these substances into common intermediates (28). In the field of the cardiac aglycones, degradation of scillaridin-A to norallocholanolic acid by Stoll, Hofmann & Helfenstein (29) has established the steroid nature of this compound. With the saponins, Tschesche & Hagedorn (30) have degraded tigogenin to aetio-allobilanic acid. Similarly, Ruzicka *et al.* (31) have established the structure of androsterone by the oxidation of *epidi*hydrocholesterol. The epimer of androsterone has been obtained by the degradation of sitosterol (32) and stigmasterol

(33), and androsterone has been obtained from cinchol (34, 35). Butenandt, Westphal & Cobler (36), and at the same time Fernholz (37), have degraded stigmasterol to progesterone, the hormone of the corpus luteum. These few examples indicate to what extent the interrelationships of the various members of the steroid group have been established in the last three years. No mention can be made of the considerable volume of work aimed at the complete synthesis of the steroids, a goal not yet attained.

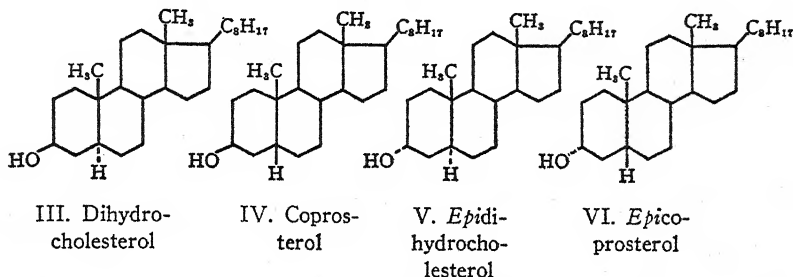
STEREoisomerism

Despite the large number of asymmetric centers in the steroid molecule (II), isomerism seems confined mainly to position 3 (hydroxyl) and to position 5 (*cis*decalin or *trans*decalin fusion of rings A and B). Windaus (39), in 1926, obtained chemical evidence for a *trans*decalin fusion of rings A and B in dihydrocholesterol and a *cis*decalin configuration in coprosterol and the bile acids. Ruzicka *et al.* (40) later obtained corroborative evidence for this from density measurements and concluded that only a *trans*decalin fusion of rings B and C and of rings C and D could be reconciled with X-ray measurements of molecular size. In all known sterols and bile acids the configurations of rings B, C, and D appear to be identical.¹

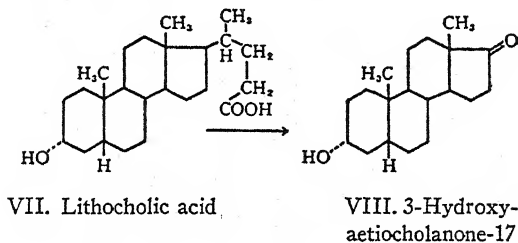
The configurations currently accepted for the carbinol group at C₃ in the sterols and their *epi* derivatives were proposed by Ruzicka *et al.* (42). They are based on the interpretation of their data, together with those of Vavon & Jakubowicz (43) and of Grasshof (44, 45), from the standpoint of the von Auwers-Skita rule. The catalytic hydrogenation of cholestanone (*trans*decalin fusion of rings A and B) in acidic medium leads to *epi*dihydrocholesterol, in neutral medium to dihydrocholesterol. Hydrogenation of coprostanone (*cis*decalin fusion of rings A and B) in acidic medium yields coprosterol; in neutral solution, *epi*coprosterol. According to the von Auwers-Skita rule, *cis* forms are usually obtained by catalytic hydrogenation in acidic media, and assuming that the configuration of carbon 5 determines the type of addition to the carbonyl group, the hydroxyl at C₃ in dihydrocholesterol (III), coprosterol (IV) and cholesterol is *cis* to the methyl group at C₁₀, while in *epi*dihydrocholesterol (V)

¹ Tschesche (41) reports that a *cis* fusion of rings B and C occurs in the cardiac aglycone, sarmentogenin.

and *epicoprosterol* (VI) it is *trans*.² All four of the isomeric cholestanols are now easily available by the catalytic reduction of the corresponding ketones in the appropriate medium.



Ruzicka & Goldberg (46) have also shown that the steric position of the hydroxyl in lithocholic acid (VII) corresponds to that in *epi*-coprosterol (*trans* to the C₁₀ methyl) by oxidizing the acid to a 3-hydroxy-aetiocholanone-17 (VIII) identical with that obtained from



epicoprosterol. As earlier work of Wieland and other investigators on the conversion of the other bile acids into lithocholic acid indicates that carbon 3 in cholic, desoxycholic, and chenodesoxycholic acids has the same configuration as in lithocholic acid, it seems probable that the steric position of the C₃ hydroxyl group in all naturally

² It seems highly advantageous to describe the configuration of the hydroxyl group as well as of other groups in terms of their steric relationship to the methyl group at C₁₀ (57). The stereochemical relation of this methyl group to the rest of the molecule is the same in all known sterols and bile acids while the hydrogen at C₅ (which is frequently used as a point of reference) may have either of two configurations or be absent entirely. The choice of the methyl group at C₁₀ as a fixed point of reference in assigning spatial configurations to other groups gives a system applicable to unsaturated sterols (such as *epi*cholesterol and *epi*-allocholesterol) as well as to the saturated sterols.

occurring bile acids corresponds to that in *epicoprosterol*, namely, it is *trans* to the methyl group at C₁₀.

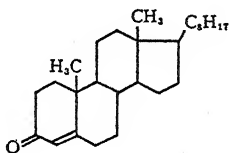
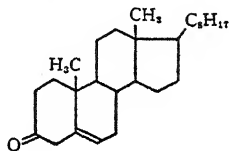
The earlier work of Windaus, according to which insoluble digonides were formed only by those sterols in which the configuration of the carbinol at C₃ corresponds to that in cholesterol, has been extended by Fernholz (47) to include steroids in which the side chain has been shortened (bile acid type) or replaced by a ketone group as in the sex hormones. None of the naturally occurring bile acids precipitates with digitonin since the steric position of the C₃ hydroxyl is that of the *epi*-sterols, e.g., *epicoprosterol*. After epimerization of the hydroxyl group, however, precipitation occurs (see also 48, 49). A similar generalization holds for the male hormones (50, 52), the epimeric pregnandiols (51) and the cardiac aglycones (144). Digitonin serves, therefore, as a valuable and convenient reagent for the differentiation of the two types of steroids arising from isomerism at C₃. The precipitation with digitonin cannot, however, be used as the sole criterion for the configuration of the carbinol group at C₃, since Butenandt & Mamoli (51) have noted several apparent exceptions. Further, the digitonin precipitability of various non-steroid substances has long been recognized (53, 54).

The epimers of the unsaturated sterols, cholesterol and *allocholesterol*, have now been prepared. Marker, Oakwood & Crooks (55), by treating the Grignard reagent from cholesteryl chloride with oxygen, obtained a mixture of cholesterol and *epicholesterol* which could be separated, either by removal of the cholesterol with digitonin or by fractional crystallization of the acetates (56). Ruzicka & Goldberg have obtained a similar mixture of cholesterol and *epicholesterol* by the reduction of $\Delta^{5:6}$ -cholestenone with the Raney catalyst (162). The epimeric *allocholesterols* have been prepared by Schoenheimer & Evans (57) by the reduction of cholestenone with aluminum isopropylate; the resulting molecular compound of *allocholesterol* and *epi-allocholesterol*⁸ was resolved by precipitation of the *allocholesterol* with digitonin. The *allocholesterol* thus obtained differed markedly in its properties from the *allocholesterol* previously described in the literature, the latter being found to be a mixture of *allocholesterol* with considerable quantities of cholesterol. Both *allocholesterol* and its epimer, on treatment with acid, are readily dehydrated to a doubly

⁸ The β -cholesterol of Diels & Linn has been found to be a molecular compound of *epi-allocholesterol* and dihydrocholesterol (158).

unsaturated hydrocarbon, $C_{27}H_{44}$, which possesses a characteristic absorption spectrum in the far ultraviolet. The ease and quantitative nature of this reaction has permitted its use for demonstrating the absence of *allocholesterol* in biological material (58). There is no evidence for the existence in nature of a $\Delta^{4:5}$ -unsaturated steroid alcohol.

$\Delta^{4:5}$ -Unsaturation does occur, however, in the naturally occurring ketones, testosterone and progesterone, both of which are analogues of $\Delta^{4:5}$ -cholestenone (IX). Butenandt & Schmidt-Thomé (59) have succeeded in preparing the $\Delta^{5:6}$ -unsaturated cholestenone (X) by

IX. $\Delta^{4:5}$ -CholestenoneX. $\Delta^{5:6}$ -Cholestenone

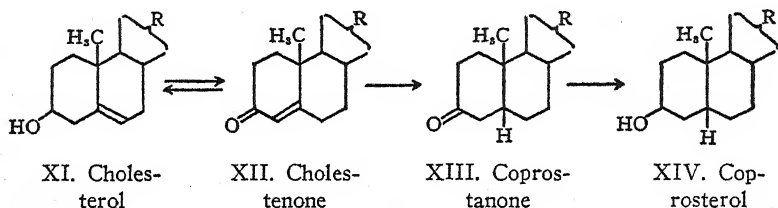
debrominating, in neutral solution, the oxidation product of cholesterol dibromide. The compound is unstable, the double bond easily shifting to the conjugated $\Delta^{4:5}$ -position in the presence of acid or alkali. The $\Delta^{5:6}$ -progesterone prepared in the same manner is biologically inactive (60). The body is apparently incapable of effecting the rearrangement so easily accomplished in the laboratory.

STEROLS

All living cells, with the exception of most of the bacteria, contain digitonin-precipitable sterols. Contrary to the results of many earlier investigations, Hecht (61, 62) has reported the presence of sterols in the tubercle bacillus and bacillus coli. Anderson *et al.* (63), working with the tubercle bacillus, could isolate only vanishingly small amounts of digitonin-precipitable material and suggest that the sterols found by Hecht originated in accidental contamination from filter paper, etc. The only species of bacteria containing significant quantities of digitonin-precipitable sterols is *Azobacter chroococcus* (64). The absence of digitonin-precipitable substances in bacteria does not exclude, of course, the possible presence of non-precipitable sterols.

Coprosterol and dihydrocholesterol.—Further information has been obtained on the biological formation of coprosterol from chole-

terol. Cholestenone (XII), when orally administered to dogs and humans, is converted into either cholesterol (XI) or coprosterol (XIV), depending upon the diet and therefore the intestinal flora



(65, 66). By use of coprostanone (XIII), in which hydrogen atoms at C_4 and C_5 had been replaced by deuterium, its conversion into coprosterol could be demonstrated by the isolation of a deuterium-containing coprosterol from the feces (65). The mechanism of the transformation is, therefore, cholestenone (XII) \longrightarrow coprostanone (XIII) \longrightarrow coprosterol (XIV), although other routes are not yet excluded. It is not yet known whether the biologically reversible conversion, cholesterol \rightleftharpoons cholestenone, occurs in the intestine or in the organism proper. By similar use of deuterium as a label it could be demonstrated that coprostanone is not an intermediate in the formation of cholic acid (67). The latter experiment indicates the need for caution in the use of chemical relationships as a basis for elaborating genetic relationships in the living organism.

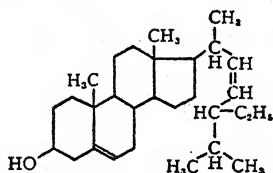
The earlier report of the presence of dihydrocholesterol in gall stones has been confirmed by Gardner & Gainsborough (68), who were unable, however, to detect this sterol in cholesterol from blood plasma.

Sterols from lower animals.—Bergmann (69) has reported the isolation of a new sterol, ostreasterol $C_{29}H_{48}O$, from oysters (*Ostrea virginica*) and from clams (*Venus mercenaria*). The compound is doubly unsaturated and on hydrogenation yields sitostanol (70). Bergmann (71) has also shown that the bombicsterol from silkworms (*Bombyx mori*) is a mixture of 85 per cent cholesterol and 15 per cent sitosterols. Bergmann & Johnson (72) have described a new sterol, microcionasterol $C_{27}H_{46}O$, from *Microcionia prolifera*, and Klenk & Diebold (73) have obtained a doubly unsaturated sterol, $C_{27}H_{44}O$, from the sea anemone, *Anemonia sulcata*.

Gillam & Heilbron (74) have found that the sterols of the lower

marine animals contain large quantities (up to 12 per cent) of a substance possessing the absorption spectrum of ergosterol. In view of the demonstrated natural occurrence of several provitamins-D (all of which possess identical absorption spectra) the identity of this substance with ergosterol cannot be assumed.

Plant sterols.—The sitosterols from wheat germ oil have been fractionated by Wallis & Fernholz (164). They have shown that the α -sitosterol of Anderson is a mixture of at least two sterols. Both compounds are doubly unsaturated and precipitable with digitonin, one being a stigmasterol isomer of the probable composition, $C_{29}H_{48}O$, the other presumably being a homologue, $C_{30}H_{50}O$. The work of Guiteras (75), Fernholz (76, 78), and Fernholz & Chakravorty (77), has elucidated the structure of stigmasterol ($C_{29}H_{50}O$). The double bonds are at $C_5:C_6$ and $C_{22}:C_{23}$. The side chain differs from that of cholesterol by the presence of an additional ethyl group at C_{24} (XV). The conversion of stigmasterol to progesterone [by Fernholz (37)



XV. Stigmasterol

and by Butenandt, Westphal & Cobler (36)] may be regarded as final proof of the assigned structure.

The identity of the tetracyclic nucleus in ergosterol and stigmasterol with that of cholesterol (33, 77) follows from their degradation to β -3-hydroxynor α llocholanic acid. The same nucleus exists in sitosterol, as evidenced by its degradation to β -3-hydroxy-aetio α llocholanone-17 (32). The position of the double bond of sitosterol from soy-bean oil has also been shown to be in the $C_5:C_6$ position (77). According to Bengtsson (79) stigmasterol and β -sitostanol are identical.

Heilbron and his collaborators (80, 81, 82) have isolated a doubly unsaturated sterol, fucosterol $C_{29}H_{48}O$, from algae. The substance on hydrogenation yields stigmasterol, and the presence of one double bond at $C_5:C_6$, as in cholesterol and stigmasterol, has been established. It is now recognized that ostreasterol, sitosterol, stigmasterol,

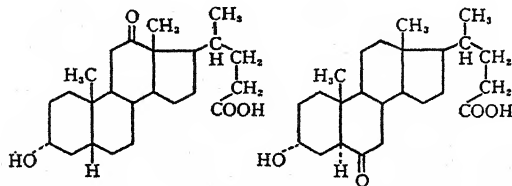
and fucosterol have a common carbon skeleton. The discovery of plant sterols or their analogues in lower animals suggests that the sharp distinction between plant and animal sterols exists only in the higher members of both groups.

BILE ACIDS

Cortese & Bauman (83, 84) have devised a new and convenient method for the preparation of conjugated bile acids. The hydroxyl groups of cholic or desoxycholic acid were protected with formyl groups and the acid chloride condensed with glycine, the formyl groups being simultaneously hydrolyzed in the alkaline medium. The synthetic glyco-desoxycholic acid has been found identical with the natural product. In contrast to the unconjugated desoxycholic acid the glyco compound forms no choleic acids. Since almost all of the bile acids in the bile are conjugated, it seems questionable whether the choleic acid principle plays a rôle in the intestinal absorption of fats and sterols.

In accordance with Ruzicka & Goldberg's (46) demonstration that the steric configuration of the bile acids corresponds to that of *epicoprosterol*, Schoenheimer & Berliner (49) have synthesized lithocholic acid (VII) from cholesterol. The epimer of lithocholic acid (β -3-hydroxycholanolic acid), which precipitates with digitonin, has been prepared by Fernholz (47) and by Reindel & Niederländer (48).

Two isomeric keto-desoxycholic acids have been isolated, both in small amounts. Wieland & Kishi (85) have found 3-hydroxy-12-keto-cholanolic acid (XVI) in cattle bile and Fernholz (86) has obtained



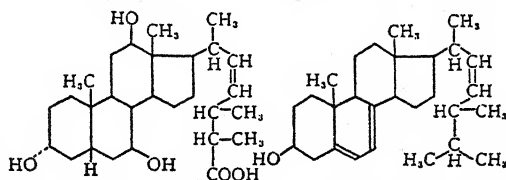
XVI. 3-Hydroxy-12-keto-cholanolic acid

XVII. 3-Hydroxy-6-ketoallocholanolic acid

3-hydroxy-6-ketoallocholanolic acid (XVII) from hogs' bile. The latter probably existed originally as a cholanolic acid derivative, conversion to

the *allo* series occurring during its isolation. Such keto acids may be regarded as possible intermediates in the formation of the corresponding polyhydroxy acids, for Yamasaki & Kyogoku (87, 88) have demonstrated the ability of the organism to reduce the carbonyl group of ketonic bile acids to the hydroxyl group.

While the representative bile acids, being C_{24} compounds, are derivatives of cholanolic acid, several bile acids containing a larger number of carbon atoms have now been found. Wieland & Kishi (85) have reported the isolation of an acid, probably $C_{28}H_{46}O_4$, from cattle bile. The substance has been assigned the name *sterocholic acid* in view of its possible relation to the sterols. Shimizu & Oda (89) have described a similar substance, trihydroxybufosterocholenic acid, $C_{28}H_{46}O_5$, obtained from toad bile. Shimizu & Kazuno (90), by ozonization, degraded the acid to trihydroxy-bisnorcholanolic acid, the toad bile acid therefore being a 3,7,12-trihydroxy acid of the structure XVIII. Iwasaki (91) has shown that the urso-desoxycholic acid



XVIII. Trihydroxybufosterocholenic acid

XIX. Ergosterol

of Shoda (92) is a stereoisomer of chenodesoxycholic acid (3,7-dihydroxycholanolic acid), the isomerism occurring at the C_7 carbinol. Lettré (93) has shown that in chenodesoxycholic acid the C_7 hydroxyl is *cis* to the methyl at C_{10} ; urso-desoxycholic acid is therefore the corresponding *trans* compound.

An acid of an entirely new type is the weak acid, $C_{29}H_{46}O_3$, obtained in small amounts from cattle bile by Wieland & Hanke (94). As its behavior towards bromine is reminiscent of that of the sapogenins, hederagenin, and oleanic acid, the compound has been named *sapocholic acid*. It may possibly be of dietary origin.

PROVITAMIN D AND VITAMIN D

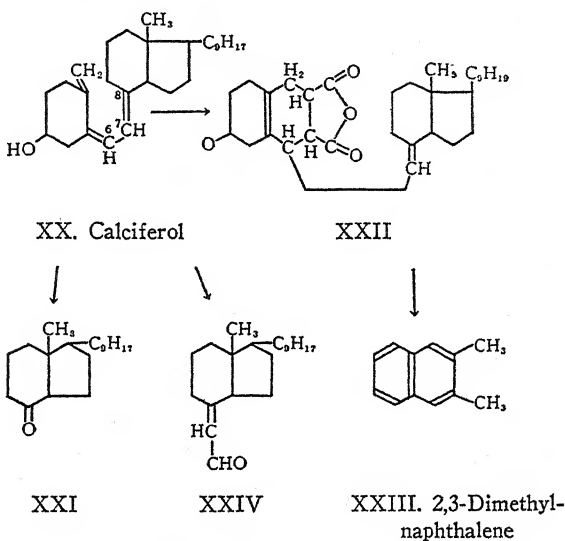
Ergosterol.—The establishment of the empirical formula for ergosterol in 1932 as $C_{28}H_{44}O$ has been quickly followed by the elucidation

tion of its structure. The position of the double bond at $C_{22}:C_{23}$ and the additional methyl group at C_{24} was early established by the isolation of methyl isopropylacetaldehyde after ozonization (95, 96). The oxidation of acetyl ergosterol to acetyl β -3-hydroxynorallocholic acid by Fernholz & Chakravorty (77) demonstrated the C_8 position of the hydroxyl and its *cis* relationship to the methyl at C_{10} ; this reaction, together with the earlier reported oxidation of ergostane to norallocholic acid (97), indicates the steroid nature of the nucleus. One of the nuclear double bonds is at $C_5:C_6$ as in cholesterol, the other is in the conjugated position at $C_7:C_8$ (XIX). This formulation is based on the following facts: The formation of neoergosterol (98) and its dehydrogenation to dehydroneoergosterol (99) indicates the presence of both nuclear double bonds in ring B. That these are conjugated follows from the absorption spectrum and molecular refraction (100), addition of maleic anhydride (101), and hydrogenation with sodium and alcohol (102). On treatment of ergosterol with perbenzoic acid (103) 1:2 addition occurs (as evidenced by oxidation with lead tetra acetate to give, on hydrolysis, a diol with one secondary and one tertiary hydroxyl group. This can only be reconciled with a $\Delta^{5,6,7,8}$ -position of the double bonds.

Vitamin D from ergosterol (D_2).—The production of vitamin D_2 [calciferol (XX)] from ergosterol on irradiation is occasioned by the opening of ring B (104) with the simultaneous introduction of a fourth double bond (105). The present formulation is based on the results of degradation experiments (106). On oxidation a ketone, $C_{19}H_{32}O$ [XXI (109)], is obtained from rings C and D, establishing the $C_7:C_8$ double bond. The selenium dehydrogenation of the maleic anhydride condensation product of the dihydro vitamin (XXII) yields 2,3-dimethylnaphthalene (XXIII), and similar reduction with platinum gives naphthalene and β -naphthoic acid, the naphthalene rings originating from the aromatization of ring A and the new ring formed by the maleic anhydride condensation at carbons 6 and 18. The aldehyde, $C_{21}H_{34}O$, obtained by Heilbron, Samant & Spring (107) from chromic acid oxidation of the vitamin, is assigned the structure XXIV (108), and the isolation of formaldehyde by ozonization of the vitamin (108, 109) supports the formulation of a methylene grouping at C_{10} .

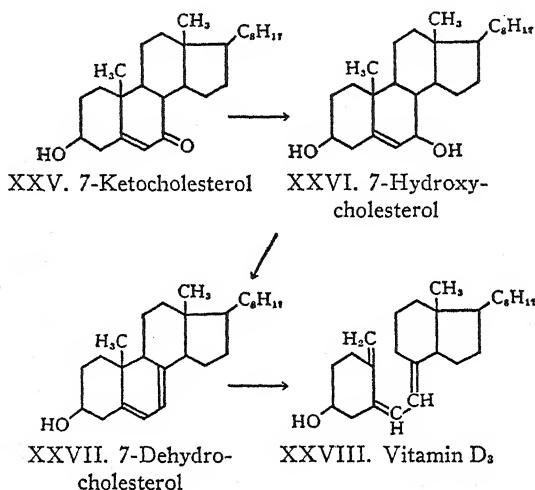
7-Dehydrocholesterol and vitamin D_3 .—During the last few years a number of investigators have made observations indicating the existence of several provitamins and vitamins D. A new light was

thrown on these investigations by the preparation of 7-dehydrocholesterol by Windaus, Lettré & Schenck (110). 7-Ketocholesterol (XXV) was reduced to 7-hydroxycholesterol (XXVI). The dibenzoate of this substance was heated in vacuo to 200° with the loss of one molecule of benzoic acid and the introduction of a second double bond at C₇:C₈ (XXVII). This substance, 7-dehydrocholesterol, possesses therefore the same nuclear arrangement of double bonds as in ergosterol and both compounds have identical ultraviolet absorption spectra.



On irradiation of 7-dehydrocholesterol a compound was isolated by methods analogous to those used with vitamin D₂ [calciferol (111)]. This compound (XXVIII) was designated vitamin D₃ and, like the vitamins from several fish oils, was found to be more active than calciferol when tested on chicks (112, 113). This chemical work was followed by the isolation of the vitamin D from tuna-fish oil by Brockmann (114) and its identification with the antirachitic irradiation product (vitamin D₃) from 7-dehydrocholesterol. This has been confirmed by Simons & Zucker (165). The provitamin present in a cholesterol sample of unspecified source has been isolated by absorption methods (115) and shown to be 7-dehydrocholesterol and not, as

was previously assumed, ergosterol.⁴ This explains the discrepancy between the assay of vitamins from various sources on rats and chicks.



The preparation of 7-dehydrocholesterol is of the greatest biological importance, not only because it is a final proof of the position of the nuclear double bonds in ergosterol but also because of its relation to cholesterol. It seems probable that the provitamin and vitamin D in the organs of higher animals are derivatives of cholesterol, rather than ergosterol.

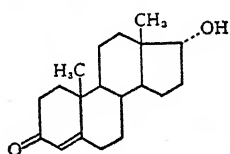
It also follows from this work that the system of conjugated double bonds in ring B is intimately connected with antirachitic activity. By introducing a C₇:C₈ double bond into stigmasterol (116) and sitosterol (117) by the methods used for cholesterol, the resulting dehydro compounds have ultraviolet absorption spectra identical with that of ergosterol. All of the known 7-dehydrosterols except 7-dehydrostigmasterol acquire antirachitic properties on irradiation, although the products differ in potency. It is not unreasonable, therefore, to anticipate the natural occurrence of a variety of provitamins in which the essential $\Delta^{5,7,8}$ -unsaturation of ring B is accompanied by differences in the side chain, etc. Haslewood & Drummond (118) have isolated from tuna-liver oil a vitamin D (*allophanate* m.p. 185–

⁴ Subsequent to the preparation of this review Windaus & Stange (163) have isolated the provitamin from cholesterol obtained from Chinese egg-powder and found it to be ergosterol.

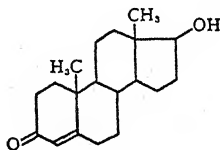
195°) apparently not identical with the vitamin D₃ (*allophanate* m.p. 173–175°) from 7-dehydrocholesterol.

SEX HORMONES

Male hormones.—During the year since the last review of this subject by Doisy & MacCorquodale⁵ efforts to correlate biological activity with chemical constitution have led to the preparation of a large number of hormone derivatives in which the original molecule has been modified in various ways. Especially striking are the variations in activity associated with modifications of the configuration of the carbon atoms in positions 3, 5 and 17. The association of increased activity with the *epi* (*trans* to methyl at C₁₀) configuration at C₃ and the *trans*decalin fusion of rings A and B in the androstane series has been previously recognized. It now appears that the configuration of the hydroxyl at C₁₇ is also important. Ruzicka & Goldberg (119) conclude, on the basis of the saponification rates of its esters, that the C₁₇-hydroxyl group in testosterone (XXIX) is *trans*



XXIX. Testosterone



XXX. Cistestosterone

to the methyl group at C₁₃. This also holds for the C₁₇-hydroxyl group of the diols predominantly formed by the reduction of androsterone and isodehydro-androsterone. The corresponding C₁₇ epimers (120) have been obtained and both the C₁₇ *cis* diol from isodehydro-androsterone and the *cistestosterone* (XXX) prepared from it are significantly less active. The epimerization of the C₁₇ hydroxyl has resulted in a striking decrease in physiological activity.

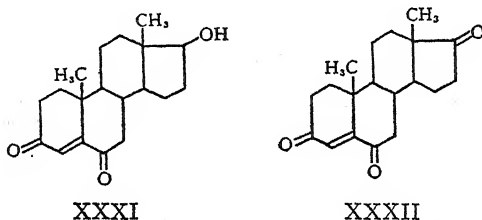
A shift of the double bond from the 4:5 to the 1:2 position occasions striking changes in physiological properties. Butenandt & Dannenberg (121) brominated androstanedione at C₂. Debromination with potassium acetate in acetic acid yielded $\Delta^{1:2}$ -androstenedione. By reduction of the C₁₇ carbonyl to a hydroxyl group, a testosterone bond-isomer with $\Delta^{1:2}$ -unsaturation was obtained. While the new

⁵ *Ann. Rev. Biochem.*, 5, 315 (1936).

compound is, like testosterone itself, an $\alpha : \beta$ unsaturated ketone, it no longer possesses male hormone activity but is oestrogenic in the Allen-Doisy test.

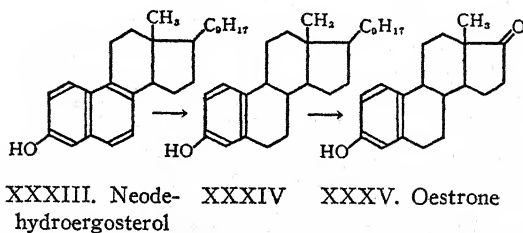
The introduction of an alkyl group at C_3 or C_{17} to yield carbinols can be effected by treatment of the corresponding C_{17} keto compounds with methyl or ethyl magnesium iodide (122, 123, 124, 125). In position 3, the reaction yields compounds of greatly attenuated activity (122); with the 17 position there is a strong enhancement of activity, the 17-methyltestosterone being physiologically more potent than testosterone itself (123).

Butenandt & Riegel (126), by the oxidation of 3,17-dihydroxy- $\Delta^{5:6}$ -androstene acetate and isodehydro-androsterone, have obtained the corresponding 3,6-diketo compounds (XXXI, XXXII). The



original materials both possess male hormone activity; the products, however, are no longer thus active but are definitely oestrogenic in the usual tests.

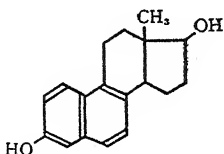
Female hormones.—The preparation of oestrone from ergosterol has been effected by Marker, Kamm, Oakwood & Laucius (38). The neoergosterol obtained from ergosterol was dehydrogenated to the naphthalenic neodehydroergosterol (XXXIII) (99). On treatment



with sodium and alcohol a tetrahydroneodehydro-ergosterol (XXXIV) was obtained. On removal of the side chain by chromic

acid oxidation, oestrone (XXXV) was obtained.⁶ Marker *et al.* (56) report the interesting observation that a mixture of epimeric 3-carboxy-androstanes possesses oestrogenic activity.

The alcoholic fraction of oestrogenic substances from mares' urine has been shown to be a complex mixture. The compound giving the color reaction of the δ -follicular hormone has been isolated in pure form (127) and shown to be a 17-dihydro-equilenin (XXXVI).



XXXVI. 17-Dihydro-equilenin

Adrenal cortical substances.—While the adrenal cortical hormone itself has not yet been isolated,⁷ the inactive crystalline substances obtained by various workers from cortical extracts (for earlier papers see past *Annual Reviews*, also 128, 129, 130, 131, 132) have been shown to be oxygenated steroid derivatives. Those possessing the general formula $C_{21}H_nO_5$ (n from 28 to 36) possess the *allopregnane* skeleton oxygenated at carbon atoms 3, 17, 20, and 21. Reichstein has definitely established the steroid nature of these compounds by demonstrating mutual interrelationships and the eventual degradation of his compounds A, C, and D to androstane (133, 134). He has also been able to isolate from cortical extracts an unsaturated $\alpha:\beta$ diketone $C_{19}H_{24}O_8$, adrenosterone, possessing male hormone activity (135).

CARDIAC AGLYCONES, TOAD POISONS, AND SAPONINS

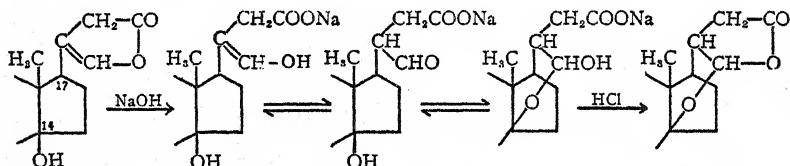
A similarity in chemical structure as well as in pharmacological action has been demonstrated for two groups of naturally occurring poisons, the glycosidic plant heart poisons and the toad poisons. In

⁶ Windaus & Deppe (166) have since reinvestigated the first stage of this reaction and report that compound XXXIV is *epi*-neosterol, i.e., that the sodium-alcohol treatment results in the hydrogenation of ring A and not ring B. On this basis it is difficult to formulate the production of oestrone by oxidation of this compound.

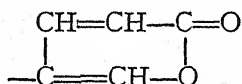
⁷ Compound H, isolated by Reichstein (131, 132), has since been found to possess cortical hormone activity (167).

both groups of compounds a steroid derivative (which is necessary for the specific physiological action on the cardiac musculature) is coupled with a non-steroid moiety. In the plant glycosides the genin or aglycone (the steroid portion) is linked with one or more molecules of sugar or sugars together with, in certain cases, acetic acid. In the toad poisons the genin is coupled with suberyl arginine.

Plant aglycones.—The aglycones of the plant heart poisons have the general formula $C_{23}H_{32-34}O_{4-8}$, with the exception of scillaridin-A from squills, which is a C_{24} compound. The steroid nature of these aglycones has been demonstrated by their selenium dehydrogenation to the Diels hydrocarbon (18, 19) and by their degradation to steroids of known structure (136, 137). The most characteristic chemical feature of the aglycones is a β : γ unsaturated lactone ring which is linked to the tetracyclic nucleus at C_{17} . This side chain is concerned in a general and irreversible isomerization of those aglycones possessing a hydroxyl group at C_{14} (138, 139). When the aglycones are dissolved in alkali the lactone ring opens and the "iso" product obtained on acidification is a saturated compound no longer giving the nitroprusside test. The reaction for the C_{23} compound may be formulated:



In scillaridin-A (C_{24}) the side chain has the structure:

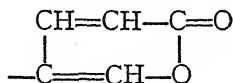


and, although an isomerization with the C_{14} hydroxyl takes place in alkali, the reaction differs from that of the C_{23} compounds (29).

The remaining oxygen atoms of the aglycones are present as hydroxyl groups attached to the tetracyclic nucleus, a $-\text{CH}_2\text{OH}$ group in ouabain and a $-\text{CHO}$ group in strophanthidin replacing the methyl group at C_{10} . Space limitation prevents a report of the experimental basis for assigning the positions of these groups. Present views as to structure are summarized in Table I. References to the large amount of earlier work are given in several monographs (3, 4, 6); the more recent literature is found in Table I.

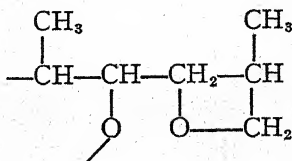
Recent work (140) has demonstrated the dependence of the physiological activity upon the steric configuration of the molecule. Epimerization of the C_8 hydroxyl is not followed by any great change in activity since thevetin, in which the C_8 hydroxyl is *cis* to the C_{10} methyl, is almost as active as its epimer, digitoxigenin. However, the *trans* fusion of rings A and B (as in α -anhydro-uzarigenin) seems to result in a striking decrease in the cardiotonic action.

Toad poisons.—Like the cardiac aglycones the toad poison genins are steroid derivatives carrying an unsaturated lactone ring at C_{17} . The steroid nature of the genin has been established by the dehydrogenation of cinobufagin to the Diels hydrocarbon (16, 17), and by the degradation of bufotalin to an isomeric cholanic acid (148). The side chain (149, 150, 151) has the probable structure:



The nature and location of the various functional groups of the compounds are as yet not exactly known. The more completely characterized genins are: bufotalin, $\text{C}_{26}\text{H}_{36}\text{O}_6$ (148, 149, 150, 152); gamma-bufogenin, $\text{C}_{24}\text{H}_{30}\text{O}_5$ (153); cinobufagin, $\text{C}_{26}\text{H}_{34}\text{O}_6$ (154); pseudo-bufotalin, $\text{C}_{26}\text{H}_{36}\text{O}_6$ (156); and bufagin, $\text{C}_{24}\text{H}_{32}\text{O}_5$ (155, 157). Since the structures offered for these compounds are as yet provisional their detailed discussion seems premature.

Saponins.—The saponins are those plant constituents of glycosidic nature which, like the soaps, form foamy, colloidal aqueous solutions. Ruzicka *et al.* (27), by selenium dehydrogenation, demonstrated their division into two groups: those in which the aglycone yields sapotalene (1,2,7-methylnaphthalene) on dehydrogenation, and a group in which a steroid genin is conjugated with sugars, as evidenced by the formation of the Diels hydrocarbon on dehydrogenation of the genin. Tschesche & Hagedorn's (30) degradation of tigogenin to actioallobilanic acid confirms the steroid nature of the nucleus. The steroid saponinins are $\text{C}_{27}\text{H}_{44}\text{O}_{3.5}$ compounds, carrying a side chain at C_{17} of the probable structure (30):



The present views as to the nature and position of the functional groups are summarized in Table II. Here again references are given only to the literature appearing since the reviews (3, 4).

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THE CHEMISTRY OF THE PROTEINS AND AMINO ACIDS*¹

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The collected papers of Sir William Bate Hardy (51) were published in 1936. His work on the proteins, which arose out of investigations of the structure of protoplasm, is of fundamental importance and its value has not been diminished by time. A brief reference may here be made to three of Hardy's discoveries, namely, the migration of albumin to the anode in alkaline solutions and to the cathode in acid solutions, the existence of an isoelectric point which is of great importance for the stability or the precipitation of the particles of denatured albumin, and the change in the point of maximum precipitation of globulin towards the acid side caused by the addition of neutral salts.

ISOELECTRIC AND ISOIONIC POINTS

The question as to whether salts can cause real changes in the isoelectric points of proteins and the possible nature of such changes has been discussed recently by a number of investigators. Hartman & Cheng (55) support the classical theory of the isoelectric point, in the form stated by Michaelis and by Loeb. According to this theory, the isoelectric point coincides with a minimum for all of the physicochemical properties of a protein or an amino acid, including cataphoresis, conductivity, solubility, osmotic pressure, swelling, and viscosity. Hartman & Cheng find, in accordance with the theory, the same value of pH 5.02 for the isoelectric point of the protein glycinin, obtained from soya bean, using four different methods: cataphoresis, solubility, viscosity, and conductivity. They refer to the careful work of Pederesen (113), who found agreement between the results obtained from measurements of cataphoresis and of solubility.

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¹ In accordance with the precedent established in previous years, this review is limited to certain aspects of protein chemistry. Much interesting work has not been discussed.

The opposite view, that the isoelectric point is a variable, is taken by Sandor (134) and by Lévy, Mignon & Netter (81), who state that the isoelectric point is affected by the buffer used for the measurement, since the charge on a colloidal particle may be affected not only by hydrogen ions but also by other ions present. These authors attach greater significance to the isoionic point, defined by Sørensen, Linderstrøm-Lang & Lund (140), which they have determined by measuring the pH values of carefully dialysed protein solutions. Theoretically, the isoionic point should be determined by measuring the pH changes caused by adding pure protein to a series of solutions of acids and bases, the point where the pH is neither increased nor diminished being determined by interpolation. If, however, the concentration of the pure protein exceeds 1 per cent, the pH value is within 0.01 units of the isoionic point in the case of the serum proteins, as shown by calculations based on titration curves (134). Similar calculations have been made for haemoglobin (7).

Sandor finds that, using collodion membranes of high permeability, practically all of the impurities can be removed from proteins by simple dialysis against distilled water. His procedure is, therefore, simpler than that of earlier workers (7, 140) who performed chemical analyses, supplemented in some cases by measurements of osmotic pressure, to determine and to allow for the effect of acidic or basic impurities. Sandor's determinations of the pH values of the solutions on successive days afford an index of the rate of purification by dialysis and of the end point. He obtains values of pH 5.10 to 5.14 for the isoionic point of horse-serum albumin at 19 to 21° C., and 5.8 to 6.0 for globulin. Lévy *et al.* (81) give the value 7.09 ± 0.18 for human haemoglobin.

Pedersen (116) has investigated the electrophoresis of the lactoglobulin of Palmer by the method, developed by Tiselius (152), of photographing the moving boundary in a quartz-U tube. This method gives a high degree of accuracy, and it has the important advantage that homogeneous proteins can be differentiated from mixtures where different fractions, or traces of impurities, migrate at different rates. The native protein gave an isoelectric point of pH 5.19 in acetate buffers of ionic strength 0.02 at 20° C. Material kept at pH 10 for one day gave quite heterogeneous curves showing that other particles were present. Lactoglobulin treated with hydrochloric acid at pH 1.8 gave uniform results but with a mobility different from that of the native protein in the same buffer mixture. Heidelberg, Pedersen &

Tiselius (60) have investigated the isoelectric points of certain antibodies.

Theorell (151) has isolated cytochrome-*c* from heart muscle and determined the isoelectric point as pH 9.82 to 9.86 in borate buffer. His curve shows rapid changes of mobility with pH over the range from 5 to 7, a region of practically constant mobility from 7 to 9, and a rapid rise from 9.5 to 10.5. The unusual form of curve obtained suggests that this protein may prove useful in testing theories concerning the relationship between the cataphoretic mobility and the acid binding power of a protein, discussed by Abramson (2).

The isoelectric points of certain mucoproteins have been investigated by Roche (121) who obtained a value of pH 3.0 for the mucoproteins of the vitreous humour of octopus, squid, and cuttlefish. A mucoprotein from octopus skin gave the value pH 3.0, whereas squid skin gave 4.0 to 4.5. A protein from snail's foot gave 3.5 to 3.8. Oldfeldt (107, 108) has investigated the titration curves of certain mucoproteins and their isoelectric points in citrate buffers. In the case of umbilical mucin, cornea mucoid, and vitreous humour mucoid, the isoionic points were pH 4 to 5, and the solubility minima pH 3.0 to 3.8. Submaxillary mucin showed a lower isoionic point, pH 3.4, and a lower pH for solubility minimum, pH 2.5. The amino acid distribution in these proteins was investigated and a comparatively low content of basic amino acids was found.

Kylin & Paulsen (78) separated three fractions from fibrinogen by cataphoresis, and observed deviations from the simple theory of the isoelectric point even in the case of the fractions. The fraction A, isoelectric at pH 5.4, had a minimum stability in alcohol precipitations at pH 7.3 and 10.1 and an optimum heat coagulation at pH 7.4. Two basic fractions were obtained, isoelectric at pH 8.5 and 12.4.

Viès (155) has described methods used for the determination of the isoelectric point of pathological sera. Debroise (27) has investigated the isoelectric point of human serum during pregnancy, and reports that, during the second month, the isoelectric points shift 2 to 3 pH units to the alkaline side. Wilkerson (159) has determined the isoelectric point of human skin powder as pH 3.7 by cataphoresis, using phthalate buffers.

The fractions of surface covered by gliadin and glutenin have been estimated from measurements of isoelectric points by Kemp (75) who has found that gliadin has an isoelectric point at pH 6.6

in dilute solution. The lower value of pH 5.1, previously reported by Kemp & Rideal (76), was obtained from material which had been allowed to stand in alcohol for some days. The measurements on glutenin yielded isoelectric points of pH 4.90 at the ionic strength 0.001 and pH 4.66 at the ionic strength 0.02.

Hagedorn, Jensen, Krarup & Wodstrup (47) have prepared sparingly soluble compounds of insulin and fish-sperm protamines, with a hypoglycaemic action lasting twice as long as the same dose of uncombined insulin, and with isoelectric points at about pH 7.3, more than two pH units more alkaline than the isoelectric point of insulin.

The question as to whether adsorbed haemoglobin has the same isoelectric point as the dissolved protein has been discussed by Dummett & Bowden (32), who reported differences, and by White & Monaghan (158), who stated that as steps were taken to remove stroma proteins by filtration and acidification to pH 5.5 the isoelectric point increased from pH 5.5 to pH 6.73. Svedberg (145) gave the value pH 6.92 at 20° C. for horse oxyhaemoglobin, determined by the moving boundary method. The conclusion that adsorbed haemoglobin has the same isoelectric point as the native protein is hardly justified by the experiments recorded, because there is no independent evidence to show that the new method for removing stroma protein is more efficient than previous methods, or to prove that the haemoglobin was unaltered by the treatment. The risk of alteration at pH 5.5 is considerable, since a loss in oxygen capacity and a diminution in the mean molecular weight have been observed at pH 5.6 (126).

Smith (137) has made an extensive study of the effects of different concentrations of cations on the apparent isoelectric point of ovalbumin, confirming the value pH 4.83 for sodium acetate buffer of ionic strength $\mu = 0.02$, recorded in a previous paper (136). The extrapolated value for $\mu = 0$ is equal to 4.85. In solutions of acetates containing the univalent ions lithium, ammonium, potassium, and sodium, the isoelectric point was found to diminish with increase in ionic strength. The minimum value of 4.7 was obtained for sodium at $\mu = 0.1$. In solutions of bivalent ions, the isoelectric pH values increase, and a maximum value of 5.4 was obtained in a solution containing the trivalent ion lanthanum.

The measurements recorded by Smith agree with the earlier comparisons of isoelectric and isoionic points made by Adair (4) and Tiselius (152) in that solutions of univalent cations diminish the iso-

electric points, although the changes observed by Smith are relatively small.

In reviewing the evidence for and against the conception of isoelectric behaviour supported by Hartman & Cheng (55), it may be noted that many observations in accordance with the theory as well as a number of exceptions have been discussed by Pauli & Valkó (111). It is possible that some apparent discrepancies may be accounted for by the presence of impurities, alterations in the proteins, and the existence of reversible dissociable systems described by Sørensen (139), which may complicate the measurements of solubility and cataphoresis. There are, however, a number of observations of variations in the isoelectric points caused by salts where the material was proved to be homogeneous, namely, the specific effects of sodium and barium acetates found by Tiselius (152), or where the method of measurement by means of membrane equilibria (7) caused no fractionation and permitted a fair comparison between isoelectric and isoionic pH values for the protein system, the isoelectric point being defined as the state where the pH value of the protein solution is equal to the pH value of the solution of crystalloids with which it is in equilibrium, and the membrane potential is zero (4).

In the case of haemoglobin at 0° C. (7), the effect of an increase in ionic strength of the phosphate buffer is represented roughly by the empirical formula, isoelectric $\text{pH} = 7.5 - 0.3\sqrt{\mu}$. Over the range from $\mu = 0.02$ to $\mu = 0.07$, the phosphates cause a shift of 0.46 pH units, whereas chlorides cause a shift of 0.1 pH units.

Smith (137) has given references to some of the hypotheses put forward to account for the effects of salts on the apparent isoelectric point. The questions at issue can be made clearer by considering more specifically the possible mechanisms by which a simple salt such as sodium chloride might diminish the isoelectric pH value: (a) The salt may diminish the activity coefficients of the ions of an amphoteric electrolyte, causing a greater diminution in the case of the cation; (b) more chloride ions than sodium ions may be attached to the particle by chemical forces; (c) the particle may be surrounded by a film of water containing an excess of chloride ions (or a deficit of sodium ions) even when the particle itself has no charge.

Electrophoretic measurements do not discriminate between these hypotheses. The first is suggested by Smith, but it involves an alteration in the isoionic point as well as the isoelectric point, and experi-

ments on glycine (17), egg albumin (140), and haemoglobin (7) indicate that the isoionic point is but slightly affected by salts.

The second hypothesis, (*b*), appears to be inconsistent with Michaelis' (92) theory that true salt formation does not alter the isoelectric point, but on the zwitterion hypothesis, a reaction of the type $\text{Na}^+ + \text{Cl}^- + {}^*\text{Pr} = [\text{Cl Pr}]^- + \text{Na}^+$ affords a possible mechanism by which the addition of a salt could give a negative charge to a protein at the isoionic point, and necessitate a fall in pH value (and the gain of a hydrogen ion) to reach the isoelectric state (6, 7).

According to the second and third hypotheses, *b* and *c*, the isoelectric protein causes a relatively greater diminution in f_{Cl} , the activity coefficient of the chloride ions, than in f_{Na} , the activity coefficient of the sodium ions. This conclusion appears to contradict the theory that the activities of individual ions have no physical significance, and Hitchcock (66) has shown theoretically that in an infinitely dilute solution, the isoelectric point could be defined without using individual ion activities. His work raises the question as to whether the changes in isoelectric point caused by salts are "apparent" or real. Although it is not possible to reach a decision on this question by pure thermodynamics, it is highly probable that the changes are real, although the results obtained by pH measurements may be subject to correction.

Although attempts to measure individual activities by means of cells with liquid junctions have been abandoned in studies of the electrochemistry of inorganic solutions, the conditions may be different in the case of protein solutions if the protein can cause marked changes in the activities without much alteration in the liquid junction potentials. The evidence obtained (6, 7) by measurements on proteins under conditions where the uncertainties due to liquid junction potentials have been reduced as far as possible indicates that haemoglobin and serum albumin cause relatively slight falls in f_{Na} , f_{K} , and f_{NH_4} , and relatively marked diminutions in f_{Cl} , $f_{\text{H}_2\text{PO}_4}$, and f_{HPO_4} , sufficient to account for the effects of these ions on the isoelectric point. In the case of the bivalent cation calcium, the data reviewed recently by Ferguson (37) are consistent with a fall in f_{Ca} , which would agree with the effects of bivalent ions on cataphoresis, noted by Tiselius (152) and Smith (137).

A number of observations have been made which support the theory that proteins form compounds with inorganic ions. Weir & Hastings (157) find that the law of mass action applies to calcium

proteinates. Abels (1) finds that the calcium-binding power of egg albumin is destroyed by acetylation and not by deamination, and infers that the hydroxyl link is of importance. References to the extensive series of investigations by C. L. A. Schmidt and his colleagues bearing on this problem have been made in previous volumes of the *Annual Review of Biochemistry*. Main & Schmidt (86) obtained evidence for complex formation in the case of manganese by a number of methods, including electrophoretic migration. The formation of carbamate compounds by amino acids and proteins has recently been considered by Roughton (129, 130) and by Stadie & O'Brien (142). Roughton has pointed out that the effects of proteins on f_{HCO_3} may exceed the effects due to carbamate formation alone. This is not inconsistent with the observation that certain compounds containing amino groups, including isoelectric glycine (142) and congo red (6), do not diminish f_{HCO_3} appreciably. Joseph (71), on the other hand, in his thermodynamical treatment of mixtures of salts and gelatin, adopts the hypothesis that the proteins "attract" the ions instead of forming compounds. The work of Loeb (83) and of Rutgers & Overbeek (131) on cataphoresis would tend to support the third hypothesis that the particles are surrounded by a film of water containing an excess of certain ions. It is not unlikely that both attraction and compound formation affect the activity coefficients of the inorganic ions.

In order to obtain experimental evidence concerning the effects of isoelectric proteins on the activities of ions (7) it is advisable to use concentrated protein solutions (20 per cent). In the dilute solutions usually employed in measurements of isoelectric points the changes may be masked by experimental errors. Analyses of protein crystals may provide evidence concerning the formation of compounds. Haemoglobin, crystallised at the isoionic point in 0.01 *M* ammonium phosphates, contained 2 mols of phosphoric acid per mol of haemoglobin (7).

THE DEFINITION AND MEASUREMENT OF ACIDITY

Hitchcock (65, 66) has suggested a new definition of acidity and a new method for its measurement. The importance of hydrogen-ion measurements in solutions of amino acids and proteins is so great that any possible improvement in this direction must receive careful consideration. The unit of acidity proposed by Hitchcock is based

on the electromotive force of a cell in which one part of the solution is in equilibrium with a hydrogen electrode and a second part of the solution is saturated with silver chloride, in equilibrium with a silver-silver-chloride electrode. The electromotive force of such a cell is given by the thermodynamic equation

$$E = E_0 - (RT/F) \ln m_{\text{H}} m_{\text{Cl}} f_{\text{H}} f_{\text{Cl}}$$

E_0 is a constant, determined by Harned & Ehlers (54); m_{H} and m_{Cl} denote the molalities of hydrogen and chloride ions and f_{H} and f_{Cl} their activity coefficients. On the assumption that the chloride is fully ionised, m_{Cl} equals the molality determined by chemical analyses, and it follows that a measurement of E , supplemented by a chemical analysis, makes it possible to evaluate the new unit of acidity $m_{\text{H}} \times (f_{\text{H}} f_{\text{Cl}})$. In the case of solutions free from chlorides it would be necessary to prepare a series of mixtures and to estimate the new unit by extrapolation.

The new unit has the advantage that it is measured by a cell without a liquid junction, where the electromotive force is determined by purely thermodynamical formulae, whereas the older unit, pH, depends upon extrathermodynamical assumptions concerning the liquid junction potentials. Even in the case of the corrected scale of $\text{p}a\text{H}$, or $-\log$ (activity of hydrogen ions), the maximum accuracy obtainable is 0.01 units (46) whereas the new unit might possibly be determined to three places.

On the other hand, there are practical difficulties which may restrict the range of application of the new unit. Studies of protein systems containing small amounts of ions would be complicated by the addition of neutral chloride. In measurements on the alkaline side of the isoelectric point, there is serious risk of secondary reactions between silver ions and proteins (111). In certain solutions, the colloid causes remarkable abnormalities in the osmotic pressures of diffusible ions of opposite sign, as discovered by Hammarsten (49). In such a solution the new unit would be affected by the activity of the protein chloride. It must be concluded that the older units of acidity are still indispensable in practical work.

Neuberger (103) has made a detailed investigation of the dissociation constants of glutamic acid and its esters, using cells with liquid junctions. His observations indicate that the uncertainty due to liquid junction potentials is small in solutions of ionic strength below 0.08,

because the apparent dissociation constants are accurate to 0.01 pH units, and their variation with the ionic strength is in accordance with the theory of Debye & Hückel. Accurate determinations of the titration curves of insulin and iodinated insulin have been made by Harrington & Neuberger (53).

Measurements of the ionisation of proteins usually represent the mean effect of a large number of groups. The solutions of methaemoglobin studied by Drabkin & Austin (29) are of special interest because the spectrophotometric measurements of the change from acid to alkaline methaemoglobin identify a special group. The results obtained at an ionic strength of 0.10 were in close agreement with the formula $\text{pH} = 8.12 + \log [\text{MetHb alk.}]/[\text{MetHb acid}]$ which is applicable to simple univalent acids. The ionisation constant is affected by the ionic strength.

Speakman & Townend (141) have determined titration curves for feather keratin and have correlated their results with the amino acid content. An extensive series of observations of the hydrogen-ion dissociation of ovalbumin have been reported by Kekwick & Cannan (73, 74) with the object of establishing a standard dissociation curve for a protein. They have evaluated the acid or base combined with the protein over a range from pH 1.5 to pH 12. The effects are reversible over the range from 2 to 11.5, but beyond this range there are slow irreversible changes. Additional measurements were made on a series of mixtures containing formaldehyde, which indicate the presence of eighteen lysine groups per mol. The assumptions concerning the activity coefficients and the liquid junction potentials that were made in the calculations have been specified. Alternative methods have been described by Sørensen, Linderstrøm-Lang & Lund (140) and by Bjerrum & Unmack (17).

The pH measurements of Kekwick & Cannan were standardised by 0.1 *N* HCl, pH 1.075, and a saturated potassium chloride liquid junction, formed in a Clark electrode vessel. The liquid junction potential with 0.1 *N* HCl is approximately 4 mv., when formed in a wide tube (17, 46). Much larger potentials have been obtained with small electrode vessels used for titrations (56). In most fairly dilute solutions over the pH range from 2.0 to 11.0, the calculated liquid junction potentials are small, rarely exceeding 1.5 mv. (17), and reproducible results can be obtained with different types of electrode vessel (56). The use of a standard liquid with a low liquid junction poten-

tial, such as $0.01\text{ }M\text{ KCl} + 0.01\text{ }M\text{ HCl}$ [Clark (21)], would facilitate the comparison of the results obtained by different workers.

THE HYDRATION OF PROTEINS

The hydration of proteins has recently been studied from a number of different points of view. The theoretical aspect is referred to by Rodebush (128) in his discussion of the hydrogen bond and co-ordination; he has stated that the problem of bound water seems likely to resolve itself as a chemical problem. He refers to the absence of an infrared band at 1.5μ in the case of gelatin as evidence for the binding of water. The co-ordination theory is supported by Jordan-Lloyd & Moran (70) who conclude that the amount of water firmly bound by gelatin (0.5 gm. per gm. of dry protein) agrees with that estimated from the number of possible co-ordination centres. The chemical theory is not accepted by all workers (72).

The question as to whether water is bound to the protein is of importance in a great variety of problems, affecting all calculations of the amounts of crystalloids bound by proteins and all estimates of the size and shape of the molecules. On account of the number of factors involved, more than one interpretation of the evidence is possible and it is not surprising that estimates of bound water show a wide range of variation. Relatively large values (exceeding 1 gm. of water per gm. of protein) have been reported in observations reviewed by Jones & Gortner (69). Moderate values (ranging from 0.2 to 0.5 gm. of water) have been obtained by a number of methods referred to by Moran (96). Evidence against the existence of any appreciable amounts of bound water has been given by Hill (64). Until recently, the argument against the existence of bound water, founded on the normal effects of added substances on the vapour pressure (64), was supported by measurements of diffusion coefficients of haemoglobin (106) and egg albumin (90) and by ultracentrifugal measurements on a number of proteins (144) which agreed in giving the same value for the diameter of the molecule as that calculated for a spherical particle with no water combined. A number of proteins gave higher values for the apparent diameter estimated by the ultracentrifuge, but these exceptions could be accounted for by the theory that the form of the molecules was not spherical. Any deviation from the spherical form reduces the rate of diffusion.

New experimental investigations of diffusion (79) and sedimen-

tation (146) have given lower values for the diffusion constants for a number of proteins including ovalbumin and haemoglobin. In the case of three proteins, insulin, Bence-Jones protein, and erythrocrucorin from *Arca* blood, the centrifugal measurements are consistent with the theory that the molecules are anhydrous and spherical, but all other cases recorded (146) are consistent with the theory that the apparent radius is increased by a combination of the effects of hydration and of deviations from the spherical form. Although absolute values for the hydration effect alone cannot be calculated, it is possible to show that a degree of hydration exceeding 0.55 gm. of water per gm. of protein would lead to larger increases in the apparent radius than many of the values recorded.

In attempting to correlate measurements of diffusion with estimations of bound water made by ordinary methods, it must be remembered that the degree of hydration may be a function of temperature, pressure, and the activities of water and other substances present in the system.

A study of the hydration of proteins in systems of two components (protein and water) has been made by Moran (96). Solutions of crystalline ovalbumin were placed in collodion sacs surrounded by ice. As the temperature fell, water passed out from the sac, and the ratio of water to albumin in a state of equilibrium was determined at temperatures down to -20°C . The following results were obtained, expressed in gm. of water (bound + free) per gm. of protein.

Temperatures	-1°C .	-2°C .	-3°C .	-5°C .	-10°C .	-20°C .
Ovalbumin	0.60	0.54	0.49	0.44	0.40	0.37
Myogen	0.70	0.67	0.62	0.57	0.52	0.46
Gelatin	1.15	0.88	0.75	0.67	0.56	0.50

Additional experiments were made on agar-agar, pure gelatin, and mixtures of albumin and gelatin, which gave (after correcting for the water bound by the gelatin) the same curve as the pure albumin. Albumin denatured by urea bound less water. In a second series of experiments albumin solutions were subjected to high pressures and it was found that at the same activity of water, the pressure method gave the same results as the freezing method. At very high pressures (2600 atmospheres) the water content fell to 0.22 gm.

Barnes & Hampton (12) have devised a method in which gelatin gels of different concentrations are frozen at -60°C ., and the presence or absence of ice crystals at higher temperatures is detected by

x-rays. They found a constant value for bound water of 0.43 gm. per gm. of dry gelatin at temperatures below -25°C. , a value quite close to results obtained by pressure and by freezing (96).

Hampton & Mennie (50) and Horn & Mennie (67) have made an important contribution to our knowledge of the thermal properties of protein systems by measuring the specific heats of gelatin gels over a temperature range from -180°C. to 25°C. , and a concentration range from 9 per cent to 100 per cent. Smooth curves with no break indicating a freezing process are obtained if the water content be below 0.477 per gm. They have attacked the problem of the determination of the specific heat of bound water. In an 87 per cent gel, the partial specific heat of water, calculated from their data by the methods of Lewis & Randall (82), is 0.61, but in the range from 60 per cent to 80 per cent the partial specific heat is abnormally high, exceeding 1.5, indicating an effect of temperature on the state of equilibrium between different forms of bound water. In gels containing less than 40 per cent gelatin it appears that the influence of the protein on the partial specific heat of water is extremely small, in spite of the solidity of the system. The specific heat measurements agree with other evidence indicating the presence of free water in gels.

Extensive studies of specific heat measurements are of value for a number of problems, such as the estimation of the free energy required to form protein from amino acids, and for the expression of the effects of the protein on the orientation of the water molecules, in terms of entropy. The entropy of hydration of simple non-electrolytes has been discussed by Butler & Reid (19).

Neurath & Bull (104) have examined the hydration of native and of denatured ovalbumin by means of density measurements, determining the contraction in volume when water is added to the dry protein. In the case of the solid proteins at 25°C. they record the densities: 1.2655_N , 1.3016_S , and 1.2940_H , where the subscripts N , S , and H denote native ovalbumin, surface-denatured albumin, and heat-denatured ovalbumin. The values for the maximum volume contraction in cmm. per gm. of material are 53_N , 30_S , and 24.5_H . The water bound by 1 gm. of protein is estimated from curves which show that the volume contraction tends to reach a constant value. The minimum water content required to reach this value is defined as the hydration (0.36 gm. per gm. of native albumin). In addition, they have given curves showing the contraction in mixtures of water and alcohol which show a minimum in the region of 30 per cent alcohol. In abso-

lute alcohol the contractions recorded are 40.0_N, 11.6_S, and 6.6_H. They advance the hypothesis that a part of the water is loosely bound, and is removed by alcohol concentrations up to 20 per cent; a second part is removed by alcohol concentrations above 55 per cent. In order to account for the difference in the density of native and denatured proteins, they suggest that the peptide chain uncoils and free spaces are eliminated.

Under the general heading of protein hydration in systems with three or more components, it is convenient to consider firstly the hydration of the protein in the form of crystals and secondly the hydration of the molecules in solution. The theoretical treatment of solutions is more complicated on account of deviations from the ideal solution laws. Recent work on the subject has been concerned almost exclusively with solutions and gels, owing to lack of data for the calculation of the hydration of the crystals; an exception is found in the case of ovalbumin in ammonium sulphate, which was studied by Sørensen (138) who gave the hydration value $W = 0.228$ gm. per gm. of dry protein. It should be stated that W represents the excess of water in the crystal, deduced from the greater ratio of water to ammonium sulphate in the crystal phase than in the mother liquor. The total water combined is equal to W if the crystal contain no salt, but is greater than W if salt be present. Recent determinations (8) of the nitrogen content of anhydrous proteins have made it possible to estimate values of W for other proteins studied by Sørensen and his colleagues, the values obtained being 0.306 for haemoglobin, 0.303 for horse-serum albumin, and 0.343 (approximately) for pseudoglobulin.

Adair & Adair (8) have given a method for the determination of the density of crystalline and coagulated proteins which affords a shorter procedure for the estimation of hydration, although in the absence of complete data concerning the specific volumes of the substances present, it is less accurate than the chemical method. The observed densities of the crystals in a medium such as sodium phosphate buffer at pH 5, namely 1.288 for edestin, 1.239 for ovalbumin, 1.237 for horse-serum albumin, 1.236 for globulin, and 1.225 for haemoglobin, are all much lower than the apparent densities of the dry proteins, which range from 1.346 to 1.329 at 20° C. The apparent density is the reciprocal of the partial specific volume of the dry protein in aqueous solution. Measurements of the partial specific volume of the protein in the crystalline state and in solution were made in

order to test the hypothesis that the difference in densities might be accounted for by a solution-contraction volume. No appreciable contraction could be detected. The alternative hypothesis, that the crystals contain water, is consistent with the data and with the observations of Neurath & Bull (104). Formulae for the calculation of the hydration, W , from measurements of density were derived and, allowing for differences in experimental conditions, the results obtained were concordant with the measurements of Sørensen. The presence of water in the protein crystals could also be demonstrated in media containing sucrose, although under these conditions the value of W was reduced. The figures ($W = 0.18$ for haemoglobin) would, however, represent minima, since corrections for the effects of combined carbohydrate must be positive.

A study of the "adsorption" of glucose by ovalbumin has been made by Gubarev & Moiseeko (45) by the method of ultrafiltration. They found that the quantity of glucose adsorbed at pH 3 was half that adsorbed at pH 8. Weber & Nachmannsohn (156) showed that at high concentrations, the "adsorption" of glucose by proteins was negative. An excess of water of approximately 0.25 gm. per gm. of serum albumin was found in the protein solutions. This apparent hydration was independent of the pH, whereas measurements of protein-crystal densities show a slight increase in apparent hydration with increase in alkalinity. This discrepancy is accounted for by the observation that the adsorption of glucose is a function of the pH (45).

The experimental investigation of the hydration of protein molecules in solutions or gels containing three or more components is complicated by the fact that the protein may combine with, attract, or repel all of the substances present in the system, and large numbers of experiments may be required to find the most suitable conditions for the estimation of hydration.

Moran (97) has made a study of the hydration of gelatin at -10° C. and -20° C. in the presence of sodium chloride, which gave values for W of about 0.33. In the presence of lithium chloride values below 0.1 were obtained. He notes that if gelatin can adsorb 0.047 gm. of sodium chloride per gm., the total hydration would be raised to 0.5, the value given for systems with two components. The term adsorption is used to represent the gross effect of all factors, chemical combination or interionic forces, by which the protein can reduce the activity of the salt. In an interesting series of experiments in which gels containing sodium chloride were subjected to pressures

of 2600 atmospheres, the water content was reduced to 0.4 gm. per gm. of gelatin and the salt content ranged from 0.008 to 0.12. At first sight the last of these values seems too high, but possible explanation may be found in the theory of Donnan & Guggenheim (28) that at very high pressures the size of the crystalloid molecules affects their distribution across a membrane.

Maizels (87) has studied the distribution of potassium and chloride ions across the wall of the red blood corpuscle, over a range of molalities from 0.1 to 0.5. At the higher concentrations, the results indicate that 10 per cent of the cell water is not free to act as solvent (about 0.2 gm. per gm. of haemoglobin). He concludes that in the more dilute solutions, which were in the range investigated by Hill (64), the effects of hydration are masked by the adsorption of salt. This conception is in accordance with observations made over a wide range of salt concentrations, which showed that haemoglobin diminishes the activity of salts in dilute solutions, up to 0.05 molal, and, if corrections for hydration be omitted, apparently increases the activity of sodium chloride in the range from 0.5 to 4.0 molal (4, 7).

Hatschek (57) has found a simple and direct method for demonstrating the presence of bound water by observing the colour change in gels containing cobaltous chloride. In the case of gelatin, from 0.44 to 0.51 gm. of water per gm. is bound in the sense that it is not available for the hydration of the salt. McBain (89) has suggested that a rigorous determination of bound water could be made by observing the sedimentation of proteins in media of different densities. The question whether his method is applicable to systems where the substance added can form compounds with the protein requires further consideration.

DIFFUSION

Lamm & Polson (79) have devised an accurate method for the determination of the diffusion of proteins, in which a transparent scale is photographed through a diffusion cell similar to that used by Tiselius & Gross (153). Their refractometric method eliminates small fluctuations due to variations in photographic blackening. Several methods for calculating the diffusion constants have been developed and applied to ovalbumin, CO-haemoglobin, serum albumin, gliadin, erythrocrucorin, and lactoglobulin. They have compared the observed and calculated distribution curves, which serve to show whether the

protein is homogeneous. These curves serve as a check on the accuracy of the experimental method in the case of homogeneous proteins. The refractive increments of the proteins were calculated from the diffusion curves and agreed with direct determinations. The molecular weights calculated from the sedimentation constants and the diffusion constants agreed with those found by the equilibrium centrifuge.

It was found that although the diffusion coefficients remained constant over the range of protein concentrations from 0.8 per cent to 3 per cent large increases were observed in the coefficients for haemoglobin, ovalbumin, and serum albumin in solutions containing less than 0.5 per cent protein. It seems possible that the protein molecules dissociate in solutions of such dilutions. In comparing the results of Lamm & Polson with those obtained by the use of the porous disc method, it appears that the "apparent radius" of the haemoglobin molecule is about 13 per cent greater than the value recorded by Northrop & Anson (106) and the difference is larger in the case of ovalbumin studied by McBain, Dawson & Barker (90).

Polson (117) has described a method for evaluating the shape and size of protein molecules from viscosity and diffusion measurements. The measurements of viscosity which give higher values than those calculated for spherical molecules are used for the computation of the ratio of the long and short axes, treating the particle as an ellipsoid; after allowances have been made for the difference in the rate of diffusion of the ellipsoid and a sphere of the same volume, it is possible to evaluate the volume of the particle. In calculating the values of the ratio, no allowances were made for hydration, and the molecular weights calculated by this method are about 70 per cent of those obtained by the sedimentation method (117). Alternative methods for determining the particle shape by viscosity and streaming double refraction have been described by Wöhlisch & Belonoschkin (160) and by Boehm (18) who includes ovalbumin and serum globulin among the particles which appear spherical.

ULTRACENTRIFUGAL MEASUREMENTS

The proteins in milk have been investigated by Pedersen (115, 116). Caseinogen was found to be present as a coarse polydisperse suspension. When brought into solution in phosphate buffers, the degree of dispersion depended on the protein concentration. The material was quite heterogeneous with regard to electrophoretic migration.

In milk serum freed from caseinogen, at least three different proteins could be identified: α , a component with a low sedimentation constant, probably the same as the protein of low molecular weight isolated by Kekwick; β , a component resembling the lactoglobulin isolated by Palmer; and γ , a protein generally called lactoglobulin. The lactoglobulin of Palmer was found to be a monodisperse protein with a molecular weight of 39,000, independent of the pH value from 1.04 to 9. At still higher pH values the protein was found to aggregate. An alteration of the sedimentation constant was observed at pH 5 and at pH 7.5, indicating a change in the shape of the particle or an alteration in hydration.

A preliminary report on the molecular state of proteins in mixtures and in concentrated solutions has further been made by Pedersen (114) who has suggested that, in the presence of clupein, serum albumin and haemoglobin dissociate into smaller molecules. The suggestion that pure proteins might dissociate at high concentrations is brought forward as a possible explanation of the high osmotic pressures of concentrated solutions. More evidence is required to compare this conception with the alternative theory that the molecular weight is constant but the osmotic coefficient increases with concentration (4).

It may be noted that Drabkin & Austin (30) found that Beer's law was valid for haemoglobin over the range from 42 per cent to one one-thousandth of this value, and they have inferred that no aggregation involving the intermolecular rearrangement of the prosthetic groups takes place. According to Schultz (135) the osmotic pressure of polymers obeys van der Waals' equation.

Lundgren (85) has observed that the dissociation of thyreoglobulin is favoured by low protein concentration, low salt concentration, and an increase in the dielectric constant caused by the addition of glycine or urea.

Annetts (11) has investigated the digestion products formed by the action of papain on ovalbumin. The protein was found to be split up into a heavy fraction, which contains no unaltered albumin, and into a light fraction which diffuses at a rate comparable with that of amino acids and polypeptides. The suggestion is made that the first stage of digestion is a loosening of bonds within the albumin molecule, followed by a splitting off of small parts from the modified protein. Heidelberger, Pedersen & Tiselius (60) have made measurements of the sedimentation of antibodies. In the case of the rabbit,

normal and immune globulins gave the same sedimentation constant, while a high sedimentation constant was given by a purified antibody prepared from horse serum.

The crystalline protein associated with the tobacco-mosaic virus has been studied by Eriksson-Quensel & Svedberg (35). At pH 6.8, about 65 per cent of the material was found to have a molecular weight of between fifteen and twenty millions. An increase or decrease of pH augmented the polydispersity and in the pH range from 4.6 to 5.5, the particles were aggregated. The protein showed uniform migration in an electric field with an isoelectric point at pH 3.49.

Svedberg & Eriksson-Quensel (147) have measured the sedimentation constant of haemocyanin in heavy water and observed a marked fall in the constant, a finding which agrees with calculations based on the viscosity and density of the solvent; the points at which the protein dissociates were found to be at a more alkaline pH in heavy water. These authors (146) have furthermore tabulated the most recent values for sedimentation and diffusion constants, molecular weights, and dissymmetry numbers. Some of the values recorded differ from those previously published; in the case of ovalbumin, a molecular weight of 43,800 is obtained from sedimentation velocity and 40,500 from sedimentation equilibria. Earlier centrifugal measurements gave the value 34,500 which, until recently, has been widely accepted as one of the best established values for the molecular weight of a protein, in good agreement with the figure calculated by Sørensen (138) from his osmotic pressure measurements and by McBain *et al.* (90) from diffusion experiments. The more recent centrifugal measurements agree with a recalculation based on Sørensen's data, made by Adair (3), which gave the value 43,000. The same figure was obtained by Marrack & Hewitt (88) from measurements of osmotic pressure. Taylor, Adair & Adair (150), using the same method, obtained the value 46,000 for material which had been recrystallised six times.

Differences of opinion have been expressed concerning the effects of solvation on calculations of molecular weights. According to McBain (89), the error due to solvation is often "insignificant but may amount to hundreds per cent." Lansing & Kraemer (80) argue that the error due to solvation must be small. Except for dilute aqueous solutions of proteins the argument is doubtful, as their mathematical treatment for systems of three components leads to the conclusion that the molecular weight of the anhydrous protein can be determined only

if the component with which the protein combines, the composition of the compound, and the partial specific volumes of both components are known. The evaluation of these terms depends upon theoretical assumptions. An exact formula which gives the molecular weight in gm. of anhydrous protein, even if the composition of the solvates formed is unknown, has been published elsewhere (5) and applied to the experimental determination of the distribution of haemocyanin in a gravitational field (127).

PLASTICITY OF PROTEINS

In the early death of Mme. A. Roche, the field of protein chemistry has suffered the loss of an able and distinguished worker. Mme. Roche's recent publications include a stimulating monograph (120) in which evidence is reviewed in favour of the recognition of a margin of variability in the constitution of certain proteins, the limit of variability constituting the degree of plasticity of a given protein. A former extensive study (119) of the effects of a prolonged protein fast in the rat had shown that under these conditions, the amounts of tyrosin, tryptophane, and particularly lysine were appreciably diminished in the muscle protein, a condition analogous to the withdrawal of arginine from salmon muscle during the spawning season. Further evidence is provided by the modification in the constitution of blood proteins under the influence of certain pathological processes, found from comparisons between two individuals or even in the case of one individual in the interval between two bleedings.

The author has recognised that the idea of a variable composition would seem to be in complete opposition to the conception of a definite protein molecule, and has discussed the possibilities of reconciliation of the two points of view. The book includes a lucid survey of some recent advances in the physical chemistry of the proteins and a discussion of Kossel's theory of protein structure in the light of modern knowledge.

Another aspect of a related problem has been studied by Roche & Roche (125) in an investigation of the colloid osmotic pressure of the haemolymph of two species of *Helix* during periods of normal conditions of temperature, nutrition, and moisture, and during aestivation and hibernation. Significant changes were noted in the osmotic pressure of the haemolymph during prolonged physiological fasts; the changes were found to persist after separation and purification

of haemocyanin and were therefore considered to represent a stable transformation of the size of the micelle rather than a transitory alteration in the state of aggregation of the protein. Haemolymph was obtained from snails maintained under controlled conditions before bleeding; in the case of *H. pisana*, osmotic pressure measurements of haemocyanin prepared after a period of aestivation yielded a mean molecular weight of 450,000, whereas the value 850,000 was determined if, prior to bleeding, the snails had existed under conditions of adequate moisture and alimentation. For the haemocyanin of a variety of *H. pomatia*, bled at the close of hibernation, a mean molecular weight of 1,000,000 was obtained, while under normal conditions the value determined was 2,400,000.

The authors have indicated the physiological significance of such changes. Haemocyanin, as the predominant protein of the haemolymph, is responsible for osmotic control as well as for oxygen carriage, and must therefore respond to the establishment of a changed osmotic equilibrium during hibernation, when, while the tissues of the snail are dehydrated, the relative water content of the haemolymph is unaffected although the total volume is reduced. An increased osmotic pressure, correlated with a diminution of molecular weight of the protein during aestivation or hibernation, would compensate the increased demands for water of the dehydrated tissues.

Changes in the composition of the serum proteins in pathological conditions have been investigated by Bierich, Lang & Rosenbohm (15, 16). Addis, Poo & Lew (9) have studied the losses of protein which take place during a fast. Luck (84) has carried out a series of experiments to investigate the question as to whether the changes in liver size and protein content, associated with differing levels of protein intake, denote the existence of a protein fraction which differs chemically from the structural proteins of the organ. His results indicate that all the liver proteins participate equally in the function of storage. The amino-acid content of liver proteins has been studied by Urban (154). Alcock (10) has reviewed the question of the synthesis of proteins *in vivo*, and has concluded that the idea that the synthesis is carried out by enzymic condensation of amino acids is untenable.

SERUM PROTEINS

Last year, the reviewer [Rimington (118)] discussed in some detail the problem as to whether the albumin and globulin fractions

which may be obtained from serum by various salting-out processes exist as individuals in untreated serum or whether they are artificially resolved constituents of native serum protein. Further information as to this question has been given by the work of Elford, Grabar & Fischer (34), who carried out the ultrafiltration of serum through graded collodion membranes of uniform porosity, and found that the simplest protein units present correspond in particle size with molecular serum albumin and pseudoglobulin, as studied individually in dilute solutions. They found also that a fraction of particle size about twice that of serum albumin was present, this being more readily retained by the membranes. The authors consider that this fraction is essentially a globulin aggregate which may correspond with the euglobulin fraction of serum. Under certain conditions, particularly upon dilution of serum with an appropriate medium, the globulin complex dissociated, whereupon the filtration behaviour of the serum protein resembled that of a mixture of the molecularly dispersed individual constituents. These results would support the view that albumin and globulin exist as distinct individuals in serum.

The experiments of Taylor *et al.* (149, 150) and of Goettsch & Kendall (42) may furthermore be considered in connection with this problem. Taylor has shown that the globulin content of normal horse serum may be determined by performing comparative titrations of anti-horse serum, containing antibodies only to serum globulin, against a standard globulin solution and against normal horse serum. The serum investigated by this method is diluted with saline but is otherwise not subjected to chemical manipulations. Goettsch & Kendall prepared antisera against human serum albumin and against human serum globulin, and carried out differential analyses of albumin and globulin in various body fluids. They obtained higher albumin/globulin ratios in lymph, oedema, and ascitic fluids than in serum, which indicates that the capillary wall is more readily permeated by albumin.

Hewitt (62) has separated two distinct fractions of widely differing properties from horse-serum albumin. After eleven recrystallisations of the albumin, he obtained a fraction which was almost colourless and practically free from carbohydrate; it yielded no humin fraction on acid hydrolysis, and had a tryptophane content of only 0.26 to 0.3 per cent, corresponding to an approximate value of 1 molecule of tryptophane to 1 molecule of albumin. A second freely-soluble fraction was obtained which was not readily heat coagulable, which contained considerable amounts of carbohydrate, and which

possessed a higher tryptophane content, a lower rotatory power, and fewer free amino groups than the crystalline fraction.

Gille (40) has published a critical discussion of methods available for the determination of euglobulin, and Roche (122) has described a micromethod for the determination of serum albumin and globulin. The precipitation of the albumin and globulin fractions in human serum by means of ammonium sulphate has been investigated by Roche, Dorier & Samuel (123, 124) who found that in the case of normal human serum, the precipitation of globulin is incomplete until 60 per cent saturation with ammonium sulphate is attained, instead of the 50 per cent commonly utilised. In the serum from certain patients suffering from arterial hypertension, 70 per cent saturation was required completely to precipitate the globulin. Samuel (133) has discussed the specificity of the characters of serum proteins in different animals in relation to the different globulin fractions obtainable and their zones of precipitation.

Studies of the colloid osmotic pressure of blood proteins include papers by Takada (148) and by v. Farkas (36). Improved forms of the Krogh-Nakazawa osmometer for the investigation of small quantities of tissue fluid have been described by Dubach & Hill (31) and by Nagaoka (99), who has carried out an extensive study of the restoration of the colloid osmotic pressure of the blood plasma of rabbits after bleeding (101, 102).

DENATURATION OF PROTEINS

Wu's theory of denaturation (161) is adopted by Mirsky & Pauling (95), who suggest that the peptide chain of the native protein is held in an uniquely defined configuration by hydrogen bonds, which are ruptured on denaturation. Wu (161) suggested that in denaturation the molecules are disrupted but remain separate, whereas in coagulation they interpenetrate. The disappearance of disulphide groups on the reversal of denaturation has been discussed by Mirsky & Anson (94). Haurowitz (58) suggests that, on denaturation, internal positive and negative groups are brought to the surface and facilitate aggregation at the isoelectric point. According to Fischer (39), denaturation is a chain reaction. Nagaoka (100) has found that a soluble form of denatured haemoglobin, prepared by heating at 52° C. at the isoelectric point, has a greater buffer value than the native protein, whereas the heat coagulated protein has a lower buffer

value. Heymann (63), by means of a sensitive dilatometer, observed an expansion of 0.0023 cc. per gm. of protein on the coagulation of ovalbumin; similar values were obtained for serum albumin and for zein. Denaturation without coagulation also increased the volume. This volume increase is correlated with changes in hydration after coagulation, estimated from densities (8) and from solution-contraction volumes (104), and with observations on the heat denaturation of albumin made by Pauli & Kölbl (110).

ISOLATIONS

A number of substances which pertain to the chemistry of amino acids and proteins have been isolated during the past year, including cytochrome-*c* by Theorell (151), crystalline trypsinogen, trypsin, and a trypsin inhibitor by Kunitz & Northrop (77), and crystalline pepsinogen by Herriot & Northrop (61). Northrop (105) has described the concentration and partial purification of bacteriophage. Eisler, Hammarsten & Theorell (33) and Dakin, Ungley & West (24) have studied the separation of blood-forming substances from the liver. Micheel & Jung (93) have obtained a highly active neurotoxin with a molecular weight of 2500 to 4000 from snake venom. Damodaram & Sivaswamy (25) have prepared a new globulin from cashew nuts and Sumner & Howell (143) have isolated a fourth crystallisable globulin from jack bean.

McCoy, Meyer & Rose (91) have isolated an α -amino- β -hydroxybutyric acid from fibrin and report that it is an essential constituent of a complete diet. Carter (20) has studied the synthesis of this acid and its isomers.

PHYSICAL PROPERTIES OF AMINO ACIDS AND PROTEINS

A comprehensive review of dielectric properties and electrical effects in solutions of amino acids and proteins has been given recently by Cohn (22). Advances made in the past year include studies of the densities and viscosities (26), the dielectric constants and electrostriction (44, 48, 59), the activity coefficients and dipole moments of amino acids and peptides (23), and the redetermination of the free energies of certain amino acids (68). It has been found that whereas surface tension is increased by glycine and alanine, it is reduced by a number of long-chain acids (109).

The solubility of muscle haemoglobin (98) and the effect of sodium chloride on the solubility of ovalbumin in 25 per cent ethanol (38) have been investigated.

According to Joseph (71), the apparent valence type of gelatin in the presence of zinc chloride is larger than the values for most proteins obtained from solubility determinations. His work includes detailed calculations of the effects of the salt on the activity of gelatin from measurements of the effects of gelatin on the activity of zinc chloride.

The calculation of activity coefficients from solubility determinations requires further consideration. In the absence of complicating factors, the effects of dilute salt solutions in diminishing the coefficient f_s , calculated from the solubility of the protein, should be concordant with their effects on a coefficient f_o which is correlated by thermodynamical formulae with the effects of the protein on the activity and membrane equilibrium of a salt, given by Adair (5) and by Joseph (71). In the case of haemoglobin in the presence of phosphates, data for the comparison of f_s and f_o are available (5, 7, 22) which show that salts cause larger changes in f_o than in f_s . The observation that protein crystals contain an excess of salt at the isoelectric point (7) indicates that the activity of the protein in the solid phase is not a constant but a function of the salt concentration.

STRUCTURE AND REACTIONS

The work of Bergmann was discussed last year by Rimington (118). Further experiments on the ratios for amino acids in blood fibrin have been carried out by Bergmann & Niemann (13) and a method for the "stepwise" degradation of polypeptides has been developed by Bergmann & Zervas (14). In their process, an amino acid is benzoylated at the α -amino group and converted into the azide through the methyl ester and hydrazide. The carbobenzoxy compound is formed on gentle heating in benzyl alcohol and hydrogenated in a hydrochloric acid solution to give the hydrochloride of the amine, which is afterwards converted to the aldehyde on warming with water. They applied this method to the degradation of the tetrapeptide glycyl-*L*-alanyl-*L*-leucyl-*L*-glutamic acid.

Grassmann & Riederle (43) obtained evidence for the existence of compounds with definite ratios of amino acids among the products of incomplete hydrolysis of collagen, one fraction being found to

contain the tripeptide lysylprolylglycine. The derivatives of kerateine, the soluble product formed by the treatment of keratin with sodium thioglycolate, have been studied by Goddard & Michaelis (41). The structure of haemoglobin has been discussed by Pauling & Coryell (112), on the basis of magnetic properties. They have concluded that the bonds from the iron atom to surrounding atoms are ionic in the case of haemoglobin and covalent in oxyhaemoglobin.

Harington & Neuberger have prepared iodinated insulin and investigated its chemical and physiological properties (53). The iodinated product was found to differ from insulin in that tyrosin units are substituted with iodine in the 3:5-positions and but little physiological activity is retained. Removal of iodine was shown to cause a considerable degree of reactivation, roughly proportional to the amount of iodine removed. This work provides additional evidence that the phenolic groups of insulin are of importance in relation to its physiological activity.

Harington & Mead (52) have synthesised glutaminylcysteine and cysteylglutamine and have compared the chemical behaviour of these substances with that of insulin. In view of the suggestion that insulin possesses an active "prosthetic" group that might contain cystine and glutamine in peptide linkage with one another, tests were made of the hypoglycaemic activity of the two peptides, but none was shown by either substance in the oxidised or reduced forms. These authors have also studied the amide nitrogen of insulin, and, by comparing the data obtained from titration curves (53) with the time course of liberation of ammonia, they have computed that insulin contains about 30 per cent of glutamic acid.

Salter & Pearson (132) have described the synthesis from thyroid di-iodotyrosin peptone of an artificial protein which relieves myxoedema.

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THE CHEMISTRY AND METABOLISM OF THE COMPOUNDS OF SULFUR*

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Newly isolated sulfur compounds.—It is interesting that as more work is done on the composition of biological material more sulfur-containing compounds are being brought to light. In addition to the relatively recent isolation of glutathione, methionine, ergothioneine, vitamin B₁, and certain sulfur-containing proteins of hormonal and of enzymic activity, there have been isolated even more recently djenkolic acid and asterubin. We have had in the past year the acceptance of heparin as a sulfur compound, and further, the isolation of a sulfur-containing substance from the adrenal cortex, the significance and origin of which arouse much interest.

The adrenal compound was isolated by Reichstein & Goldschmidt (1) from the saponified petroleum ether-soluble fatty fraction of an extract of the adrenal cortex. The compound has the empirical formula, C₄H₁₀O₃S, and from its chemical behavior the structural formula was suspected to be HOCH₂ · CH₂ · SO · CH₂ · CH₂OH. Synthesis was effected by the oxidation of bis-(β-hydroxyethyl) sulfide with hydrogen peroxide and the properties of the synthetic material proved to be identical with those of the isolated compound. As the authors point out it cannot be concluded from the data now available whether or not the compound exists in the tissues as the sulfoxide. It appears to be associated with fatty acid and the indications are that it does not belong to any of the principles of the adrenal cortex which possess hormonal activity.

The work that has accumulated concerning heparin makes it seem unquestionable that this compound contains sulfur, thus justifying the claims of Jorpes (2). The crystalline preparation of heparin which has been isolated by Charles & Scott (3) has now been shown to contain the sulfuric acid ester grouping. The heparin-like activity of a number of sulfuric acid esters of natural polysaccharides is also of interest in this connection (4). The most recent view of Jorpes &

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Bergström (5) is that heparin resembles a mucoitin sulfuric acid in contrast to their previously expressed view that it behaved like a chondroitin derivative.

Synthetic studies.—In last year's *Review* the isolation, by Van Veen & Hyman, of djenkolic acid from the urine of individuals suffering from djenkol poisoning, was reported. On the basis of analytical data and chemical behavior these authors suggested the following structural formula: $\text{CH}_2(\text{SCH}_2 \cdot \text{CHNH}_2 \cdot \text{COOH})_2$ (6). This has now been established by the synthesis of the compound by du Vigneaud & Patterson (7). This cysteine thioacetal of formaldehyde was prepared by the addition of methylene chloride to the sodium salt of cysteine in liquid ammonia. The synthetic product was shown to be identical to the naturally-occurring djenkolic acid by a direct comparison of the two compounds.

The reductions of dicarbobenzoxycystinyldiglycine and of S-benzylcysteinylglycine to cysteinylglycine by metallic sodium in liquid ammonia (8) have been utilized as key reactions in a new synthesis of glutathione by du Vigneaud & Miller (9): the methyl ester of S-benzylcysteinylglycine was condensed with the acid chloride of α -methyl-N-carbobenzoxyglutamate; after saponification of the condensation product, the N-carbobenzoxy- γ -glutamyl-S-benzylcysteinylglycine so obtained was reduced with sodium in liquid ammonia, yielding glutathione. The yields obtained in the various reactions and the reagents and procedures involved were of such a nature that the synthesis holds much promise as a preparative method where large amounts of the compound are required.

The synthesis of cysteinylglutamine and glutaminylcysteine has now been reported by Harington & Mead (10). Neither peptide in the reduced or oxidized form possessed any hypoglycemic activity. The amide nitrogen was found to be quite labile but the sulfur was found to be more stable than the sulfur of insulin. An interesting differential reduction was utilized in the synthesis of the glutaminylcysteine: It was found that the benzyl ester grouping is removed by reduction with phosphonium iodide much more slowly than the carbobenzoxy group. It was also indicated that the S-benzyl group is affected even more slowly.

A new synthesis of methionine by Hill & Robson (11) from α -benzoylamino- γ -butyrolactone has been described which is of interest in that it calls attention to a possible relationship between certain of the amino acids. The authors suggest that the aminolactone

might be the immediate precursor of methionine in nature, and perhaps the starting material for the natural synthesis of aspartic acid, glutamic acid, ornithine, arginine and substituted glyoxalines. The idea of such a relationship is indeed a very stimulating one, but as admitted by the authors neither the lactone nor the α -amino- γ -hydroxybutyric acid has as yet been found in nature.

Because of the increasing interest which has been shown recently in the metabolic behavior of N-methyl derivatives of essential amino acids as a possible means of affording information relative to the steps in the metabolism of the corresponding amino acids, Patterson, Dyer & du Vigneaud (12) have synthesized di-N-methylhomocystine and N-methylmethionine.

Practical details have been described by Cortese (13) for a convenient and inexpensive preparation of taurine from β -aminoethyl bromide hydrobromide, while White & Fishman (14) have very recently described conditions for the successful repetition of Friedmann's (15) transformation of cystine into taurine. The melting point of pure taurine was found (14) to be considerably higher than that recorded in the literature. The success of this preparation of taurine from cystine assumes added interest because of the difficulty experienced by others in repeating Friedmann's synthesis.

The preparation of numerous additional sulfur-containing organic compounds has been described during the past year but only those that seem to be of immediate biochemical significance have been considered here.

Chemical behavior.—Herbst (16) has now brought forth evidence to explain the production of acetaldehyde in the reaction of cystine with pyruvic acid. The new work still assumes the intermediary formation of a Schiff base between α -amino acids and α -ketonic acids but in the shifting of the hydrogen atom from one moiety to the other carbon dioxide may be split off from either the ketonic or the amino acid residue, depending upon the other groups present. In the case of the Schiff base of cystine and pyruvic acid, acetaldehyde and cystine would result if carbon dioxide were split from the pyruvic acid moiety while thioglycolaldehyde and alanine would result, theoretically, from the alternative decomposition. Apparently the acetaldehyde arises from the pyruvic acid by way of the first pathway just mentioned and does not arise from the cystine as originally postulated.

The isolation of the disulfoxide of *l*-cystine by Lavine & Toennies

(17) was reported in the *Review* last year.¹ The detailed account of the method of preparation has now been presented (18), and Lavine (19) has studied further the reactions of this compound, especially its dismutative decomposition. While certain reactions with thiol compounds and with sodium cyanide strongly suggested the structure of the disulfoxide to be that of a thiol sulfonate, $RS \cdot SO_2 \cdot R$, the ease of its reduction with aqueous KI-HCl strongly suggested the stability of the fully saturated sulfur atom of the disulfoxide structure, $R(SO)_2R$. It was pointed out, however, that there may be an equilibrium between the two forms of the disulfoxide.

In two detailed reports Kharasch, Legault, Wilder & Gerard (20, 21) have reported data which have accumulated from their exploration of the catalytic action of iron, copper and manganese ions on the oxidation of thioglycolic acid. The effects of temperature, oxygen tension, initial thiol concentration, initial dithiol concentration, hydrogen ion concentration, character of buffer, combination of metal ions, and the effects of certain inhibitors of catalysis, such as pure phosphate, cyanide, and other substances, have received detailed consideration. They have also considered the ability of tissue extracts to enhance or decrease the rate of the metal-catalyzed oxidation of thioglycolic acid.

Schöberl & Eck (22) in a continuation of their study of the hydrolytic fission of the disulfide linkage have emphasized the fact that compounds of the type $[RCH(COOH)S]_2$ give rise to one molecule of the corresponding sulfhydryl compound and one of the corresponding sulfenic acid. The latter then splits out hydrogen sulfide to form $R \cdot CO \cdot COOH$. If the R group is H, glyoxylic acid is formed while if R is CH_3 , pyruvic acid is formed. They have also shown that if R is COOH or $CH_2 \cdot COOH$ the compound loses carbon dioxide to yield glyoxylic acid or pyruvic acid. Two molecules of the sulfenic acid can also yield one molecule of the sulfhydryl and one molecule of sulfinic acid. The ease of hydrolysis of the disulfide linkage may be significant with respect to the physiological behavior of these compounds and some of the conflicting observations in regard to the effects of sulfhydryl and disulfide compounds on enzymes may be due to this hydrolytic decomposition having been overlooked.

The decomposition of cystine dimethyl ester which takes place

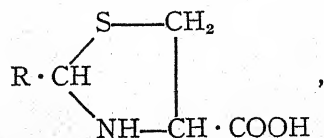
¹ *Ann. Rev. Biochem.*, 5, 162 (1936).

slowly at room temperature has been studied by Coghill (23). It was shown that sulfur, ammonium sulfate, *dl*-alanine anhydride and two other products of complicated and unknown nature were formed. He has suggested that the course of this decomposition is through cystine anhydride.

Toennies, Lavine & Bennett (24) have determined, under carefully controlled conditions, the specific rotation of cystine of 98 to 100 per cent purity at acidities varying from pH 0 to 12.0. The values obtained on this pure material have served as reference data for the determination of the stereochemical purity of *l*-cysteine. Because of the far greater specific rotation of cystine in comparison with that of cysteine, the oxidation of the latter to cystine gives a far more sensitive criterion of the stereochemical purity of the *l*-cysteine (25).

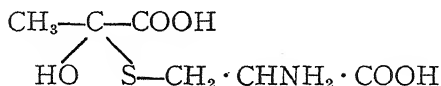
Because of the suggestion that the inhibition of glycolysis by iodoethyl alcohol is due to a mechanism other than an interaction with thiol groups of the enzyme, Goddard & Schubert (26) studied the reaction of iodoethyl alcohol with cysteine and succeeded in the isolation of S-(β -hydroxyethyl)cysteine. They also demonstrated that iodoethyl alcohol reacts with glutathione and reduced proteins. It was, therefore, concluded that while the rate of reaction of iodoethyl alcohol with sulfhydryl is slower than that of iodoacetic acid, the reaction does occur and there is no need to assume different mechanisms of inhibition of glycolysis by iodoacetic acid and by iodoethyl alcohol. The kinetics of the reaction between iodoacetic acid and cysteine and glutathione have been studied by Rapkine (27). Smythe (28) has compared the relative effects of iodoacetate and of iodoacetamide on various sulfhydryl compounds and has shown that the reaction in each case is more rapid with iodoacetamide than with iodoacetate.

Schubert (29) has observed that the interaction of thiols and carbonyl compounds is much more general than was at first suspected. Cysteine was found to give condensation products with simple aldehydes having most probably the structure



with the exception of the cysteine-pyruvic acid compound which is

formed by the simple addition of these two acids to give what is thought to be



Schubert suggests that these aldehydethiol compounds may be biochemically significant because of their reversible formation and dissociation. These chemical observations are of interest in relation to the mechanism of the natural synthesis of djenkolic acid which Van Veen & Hyman (6) suggested might occur in the plant through a condensation of formaldehyde with cysteine. Apparently the thioacetals such as djenkolic acid are not formed under the conditions employed by Schubert. Friedmann & Giršavičius (30), in studying the reactions of pyruvic acid with thiolacetic acid and with cysteine, agree in most part with the observations of Schubert but they feel that the nature of the compound which is formed in the reaction between pyruvic acid and cysteine is still to be settled.

Chemical behavior of the sulfur of proteins.—The behavior and possible significance of the sulfur moieties of proteins and protein-like substances have been considered from numerous aspects.

Mirsky, and Mirsky & Anson, in a series of papers, have presented some of their fundamental studies concerning the sulphydryl and disulfide groups of proteins. In native egg albumin no sulphydryl groups were detectable while in completely coagulated albumin as many groups were detectable as were found in the hydrolyzed protein (31). In the reversal of denaturation of serum albumin, when the insoluble protein regained its solubility, disulfide groups which had been detected in the denatured protein could not be detected after reversal. In the case of the proteins of hemoglobin and of the crystalline lens (32) a different picture was presented, for active sulphydryl groups were found in these proteins in the native state. It might be pointed out that in this paper the authors report that sulfuric acid stronger than 6 N yielded higher cystine values with the Folin-Marenzi method than when 6 N was used and they suggest that with 6 N sulfuric acid substances that interfered with the estimation of cystine by the Folin-Marenzi method may have been formed. The possible conversion of methionine to homocysteine under these conditions was not mentioned. In a later paper Mirsky (33) reports that the native proteins of minced muscle also contain sulphydryl groups. When myosin was exposed to a typical denaturing agent such as acid

it became insoluble and an increased number of sulfhydryl groups became detectable, whereas myosin which had become insoluble because of the onset of rigor was found to contain the same number of active sulfhydryl groups as the soluble myosin of resting muscle (34). It appears that protein coagulation as brought about by heat, surface forces, ultraviolet radiation and various chemical agents is distinctly different from the coagulation of myosin during rigor. All of the work of Mirsky and Anson indicates that the process of denaturation is intimately bound up with the reactivity or availability for reaction of the sulfhydryl and disulfide groups. The studies are, furthermore, in harmony with the view that denaturation is a definite chemical reaction and that a protein molecule is either native or denatured. The thesis that the denaturation of some proteins can be reversed is strongly supported by the work.

Very significant and interesting work has appeared during the past year on the chemical behavior of the sulfur of wool. Smith & Harris (35, 36, 37) in a series of studies relating to the oxidation of wool have shown that the main point of attack of both hydrogen peroxide and of the photochemical oxidation by irradiation is the disulfide group of the cystine in the wool. The experimental findings suggest that hydrogen peroxide bleaching causes a partial oxidation of the cystine sulfur of the wool without lysis of the disulfide group. The results of their irradiation studies indicate that the photochemical decomposition of wool involves an oxidation reaction but that the mechanism of this oxidation must be very different from that of the oxidation by such a reagent as hydrogen peroxide. Crowder & Harris (38) suggest that the behavior of the sulfur in the wool during alkaline degradation might be explained by the mechanism advanced by Schöberl & Wiesner (39) to explain the sulfur lability of disulfides in alkaline solution.

Work has also been reported by Schöberl (40) on the lability of the sulfur of sheep's wool. He, too, has come to the conclusion that the mechanism of the hydrogen sulfide production is through the degradation of an unstable sulfenic acid which results from the hydrolytic cleavage of the disulfide. This accords with his earlier postulation for disulfides foreign to the protein molecule. In this report Schöberl has discussed this mechanism at length as a possible explanation of the production of hydrogen sulfide, ammonia, and pyruvic acid by the alkaline treatment of cystine.

Andrews (41) has demonstrated the interesting fact that glass

wool has a catalytic effect on the hydrolysis of hair by hydrochloric acid. He also found that in hydrochloric acid and phosphoric acid the hair was physically disintegrated at 38° in a few days' time, while in sulfuric acid after three years the hair still retained its normal physical appearance. The liquid portion contained no free cystine.

That the cystine is changed more or less while the sulfur is left relatively unchanged in the deamination of proteins by the method of Dunn & Lewis (42) has been indicated by the work of Hess & Sullivan (43).

In digestion studies *in vitro* Jones & Gersdorff (44) have followed the liberation of cystine by the Sullivan method in the tryptic digestion of casein. The cystine so liberated was largely destroyed in the presence of casein and trypsin at a pH of 8 to 9 and this destruction was shown to be greater in the presence of the trypsin. The cystine liberation in a pepsin digest of uncoagulated crystalline egg albumin has been followed by Calvery, Block & Schock (45) by the Folin-Marenzi method. An apparent maximum value after twelve hours' digestion, followed by a drop to a lower constant value, was observed. Jones and coworkers (46) had observed similar behavior in the early stages of the hydrolysis of casein with pepsin and with acid.

Methods of determination.—A number of modifications of the standard methods for determining sulfur compounds of biological interest have been described. One is left with the impression that difficulties are apt to be encountered frequently in the application of any of the methods to complex biological materials. While comparative studies may be entirely successful one should be extremely conservative in the acceptance of values obtained as representing actual values. This feeling of uncertainty is increased by the controversial and contradictory results that are appearing from different laboratories in applications of the same methods to the analysis of similar materials.

A new procedure for the determination of methionine in proteins has been proposed by Baernstein. The method is based on demethylation of methionine and oxidation of the resulting homocysteine with sodium tetrathionate (47). Combined methods for the determination of cystine, methionine, and sulfates in hydriodic acid digests have also been outlined by him (48).

Sullivan & Hess (49) have applied the Sullivan test for cystine to the determination of cystine in urine. Interfering substances are taken care of by treatment of the urine with a large amount of alkaline sodium cyanide. They emphasize, in the general application of

the method, the need of using extra amounts of alkali in the presence of highly buffered materials and of the need of greater concentrations of the naphthoquinone reagent when determinations are made in the presence of strong oxidizing or reducing contaminants. A critical study of the Sullivan method for the determination of cystine from the standpoint of possible inhibitors of the reaction has been made by Andrews & Andrews (50).

Medes (51) has also presented a method for the determination of the cystine content of normal urine in which precipitation with cuprous chloride is followed by recovery of cysteine by decomposition of the cuprous mercaptide with hydrogen sulfide and subsequent quantitative determination of the cysteine with phospho-18-tungstic acid. The results obtained by this method and various other methods (52, 53) which she applied to the urine suggested to her the possibility that some other disulfide compound such as homocystine may be present in urine in small amounts.

Additional papers have been published by Shinohara & Padis in the series of studies on the determination of thiol and disulfide compounds. The investigations have now resulted in a method for determining the purity of cysteine preparations by quantitative inhibition of the Folin-Marenzi cysteine color production with mercuric chloride (54). A detailed study has been made of the Folin-Marenzi method for cystine determination (55). A method for the quantitative differentiation of cysteine or cystine from ascorbic acid has also been presented (56), and the application of this method to the determination of urinary cysteine, cystine, and ascorbic acid has been described (57).

A modified nitroprusside method for the determination of reduced glutathione has been outlined by Zimmet & Dubois-Ferrière (58) who employ nickel trichloride in sodium nitroprusside and obtain a lilac-rose colored precipitate with glutathione. This reagent is said to be superior to the ordinary nitroprusside reagent in both sensitivity and stability of the end product. This use of nickel trichloride to increase stability of the nitroprusside reaction suggests to the reviewers a resemblance to the stabilizing effect of zinc acetate upon this reaction as observed by Giroud & Bulliard (59).

In a comparison of the Benedict-Denis and the Parr bomb methods for the determination of the total sulfur in proteins, Painter & Franke (60) have observed that the Parr bomb method yields higher values. In investigating the behavior of the individual sulfur-con-

taining amino acids in these determinations it was found that the value for cystine sulfur by the Benedict-Denis method was 96.5 per cent while that for methionine sulfur was only 36.8 per cent of the true value.

Stekol (61) has recently described two methods for the determination of *p*-bromophenylmercapturic acid in the urine of the dog. In one the mercapturic acid is decomposed into *p*-bromophenylmercaptan which is precipitated by mercuric chloride as a mercury complex and weighed. In the other method the *p*-bromophenylmercapturic acid is titrated with standard iodine solution.

Metabolic investigations.—One of the most significant metabolic studies which has been reported during this period is that of Rose and coworkers (62) which, while confirming the observation of Jackson & Block (63) that methionine can replace *l*-cystine for growth in the diet of rats, has brought out the important fact that *l*-cystine will not serve for growth in lieu of methionine in the diet of animals on a cystine-methionine-deficient diet. Methionine, therefore, is shown for the first time to belong to that group of amino acids regarded as being "essential" in the diet. Confirmation of the methionine utilization in lieu of cystine in this instance is unusually significant, since a mixture of pure amino acids was used to replace the customary proteins, and the diets were thus devoid of cystine except for small amounts which were present in the vitamin supplements. The isolation of threonine by Rose and coworkers (64) has brought closer the realization of a "perfect" diet for such growth studies. The final goal will be reached when the nutritional and chemical knowledge of the vitamins is such that they can likewise be furnished in chemically pure form and hence the inclusion of any extraneous cystine or methionine in the diet can be avoided.

An interesting observation concerning the physiological behavior of methionine and cystine has been made by Weichselbaum (65). When young rats were maintained for a long period on the Sherman-Merrill cystine-deficient diet, many developed a peculiar series of symptoms and died during the sixth week of the experiment. The characteristic symptoms could be prevented by cystine or methionine but once the pathological effects were established they could be relieved by cystine but not by methionine. In the light of the findings of Rose and his colleagues (62) it must be concluded that sufficient methionine was present in the Sherman-Merrill diet since growth was resumed when cystine was fed, but an insufficient amount of

methionine to supply the need for both methionine and cystine was present. This comment applies equally well to all diets used hitherto in cystine-deficiency studies in which the addition of cystine alone promoted growth. The failure of methionine to show the same effect as cystine in Weichselbaum's studies would indicate that this curative effect may be dependent upon the presence in the organism of a high concentration of disulfide or sulfhydryl material or that the animal has lost the ability to transform methionine to cystine while on the diet.

Growth studies with N-methylhomocystine and N-methylmethionine (12) have demonstrated that both of these methylamino acids are able to support the growth of animals on a cystine-deficient diet. The utilization of these amino acids through deamination to the keto acid was discussed and it was inferred that the keto acids of these sulfur-containing amino acids can be utilized by animals in place of the corresponding amino acids.

Hayward, Steenbock & Bohstedt (66) have now shown that raw soybeans support growth poorly but that if the beans are autoclaved the nutritive value of the protein is practically doubled. The same beneficial effect could be obtained by supplementing the raw soybeans with *l*-cystine or casein supplements. Apparently cystine or its equivalent may exist in the raw protein of the soybean in a form which is not available to the animal and heating the soybean causes the cystine fraction of the protein to become available. Although the possibility also exists of a toxic factor being present which may be detoxified by heating or by the use of excess cystine in the diet, the remote possibility might well be considered that part of the cysteine sulfur of soybeans may occur in the form of a thioacetal complex such as djenkolic acid in the djenkol bean. Whether or not the heat treatment which appears to make available for growth the cystine of the soybean would also make available the cysteine of the thioacetal complex in the djenkol bean might be profitably investigated.

In a continuation of their studies with djenkolic acid Hyman & Van Veen (67) have found that it is as well oxidized as cystine in rabbits maintained on a constant basal diet. They also observed that normal persons were able to oxidize djenkolic acid, while "djenkolic patients" showed individual variations in their ability to oxidize this substance. Growth studies indicated that this acid would not serve for cystine in a cystine-deficient diet. In an analysis of their results, however, Van Veen & Hyman conclude that the djenkolic acid might

have been available but that it might have been toxic to the kidney and thus have masked the utilization effect. While the reviewers realize that such an explanation of the experimental observations could be correct, they feel from studies made in their own laboratory with the synthetic *l*-djenkolic acid (68) that the animal organism is unable to free cysteine as such from its thioacetal of formaldehyde.

Jackson & Block (69) have reported that dithioethylamine fails to replace cystine in the diet of rats for the purpose of growth. This is in agreement with the negative results of Mitchell's investigation (70).

It has been demonstrated by Dyer & du Vigneaud (71) that glutathione, whether administered orally or injected subcutaneously, is able to support the growth of rats on a cystine-deficient diet, thus indicating the possibility that cystine or cysteine can be liberated from the tripeptide during metabolism. It may be recalled that doubt concerning the cleavage of glutathione to its constituent amino acids had resulted from enzyme experiments which indicated a resistance of this γ -amino tripeptide to complete hydrolysis by proteolytic enzymes (72) and from the observation that cysteine administered to cystinuric subjects gave rise to extra urinary cystine while glutathione caused practically no increase (73).

In close relationship to the above finding is the observation by Voegtlin, Johnson & Thompson (74) that the growth of a typical malignant tumor of mice was slowed or even inhibited when the animals were fed on a cystine-deficient diet but when glutathione was injected subcutaneously into the animal host, or when cystine was given orally, the rate of growth of the tumor was accelerated. Since it was shown by them that cystine supplements to this basal diet would stimulate the growth of young mice, and since glutathione had been shown (71) to support the growth of animals on a cystine-deficient diet, Voegtlin and coworkers conclude that there is no essential difference between normal growth of young mice and rats on the one hand and the growth of the spontaneous mammary carcinoma on the other. The mechanism responsible for the stimulating effect of glutathione on malignant growths was discussed.

Reference should also be made to an interesting turn of events in the studies on the relationship of the thymus gland to growth and development. The recent work of Rowntree and coworkers (75) foreshadows the possibility that glutathione may be one of the factors

responsible for the production of precocity in rats when thymus extracts are administered to the preceding successive generations.

Akobe (76) has described experiments to demonstrate that both *d*- and *l*-hydroxymethionine will support the growth of rats maintained on a cystine- and methionine-deficient diet. The data, however, are not quite as conclusive as might be hoped, but no doubt the investigators were hampered by the difficulties involved in obtaining the active hydroxy acids.

A novel approach to the study of cystine and taurine metabolism has been initiated by White (77) who has produced an apparent cystine deficiency in rats by the feeding of cholic acid. Animals receiving the cholic acid ceased growing and gradually lost weight. The cessation of growth was due apparently to an exhaustion of the cystine or methionine essential for nutritional purposes since resumption of growth resulted from the administration of *l*-cystine or *dl*-methionine or the withdrawal of the cholic acid. Administration of taurine did not support growth. The last observation lends support to the suggestion of Lewis (78) that the formation of this bile acid in the organism occurs subsequent to the conjugation of cholic acid with cystine.

In striving to advance the knowledge of the physiological origin of asterubin, Ackermann (79) has investigated the possibility of taurocyamine $[\text{HN}:\text{C}(\text{NHCH}_2\cdot\text{CH}_2\cdot\text{SO}_3\text{H})\text{NH}_2]$ serving as a precursor of asterubin $[\text{HN}:\text{C}(\text{NHCH}_2\cdot\text{CH}_2\cdot\text{SO}_3\text{H})\text{NMe}_2]$. No evidence of methylation occurred.

The question of absorption is of significance to most metabolic investigations and the continued studies of Andrews and coworkers (80) on the absorption of various sulfur-containing amino acids from Thiry jejunal loops of dogs is, therefore, of considerable interest. The observation that significant changes occurred during three years of experimental studies in the rates of absorption of cystine and cysteic acid from the same loop is a point not to be overlooked.

Of interest also to the question of absorption is the observation of Linderstrøm-Lang & Duspiva (81) concerning the ability of the larvae of certain insects to utilize keratin which is resistant to digestion by known proteolytic enzymes. The work shows that the larvae of *Tineola* possess an intestinal reducing agent which changes the keratin to keratein and so makes it susceptible to a special protease called keratinase. This enzyme differs from trypsin-kinase in being active in the presence of sulfhydryl groups. It may be recalled that

Grassmann and his coworkers (82) have shown that trypsin-kinase is strongly inhibited by thiol compounds.

Of much importance to the physiological significance of glutathione may be the *in vitro* studies of Hopkins & Morgan (83) on the interrelationship of ascorbic acid and glutathione in the presence of hexoxidase of plant extracts. Glutathione was found to protect the vitamin from oxidation by the hexoxidase while it was itself oxidized at a rate which was exactly the same as the rate with which ascorbic acid was oxidized alone. Reversibly oxidized ascorbic acid, moreover, was very difficult to reduce by glutathione alone, but in the presence of the enzyme the rate of reduction was greatly increased. The tripeptide also was found to completely protect ascorbic acid from oxidation by copper catalysis but the mechanism appears to be different from that which operates in the case of the enzyme.

Cystinuria.—During the period of this review few real advances towards a final solution of this anomaly have been made although it is hoped that current investigations will provide in the near future conclusive evidence as to its mechanism and will produce some means of alleviation of the symptoms. Lewis, Brown & White (84) have reported that their findings with the oral administration of methionine, cysteine hydrochloride, and cystine to a cystinuric male are in general confirmation of Brand and coworkers (73) in so far as they had found an increase in urinary cystine after the ingestion of methionine and cysteine. The increases in cystine excretion observed by them were not as great as those observed by Brand's group. As pointed out by Lewis *et al.*, they themselves had used the Lugg-Sullivan method whereas the most striking results of Brand appeared to be calculated from the values obtained by the Folin method, a method which Brand has subsequently shown (85) to give results somewhat too high in the presence of the demethylation products of methionine. The possibility of a difference in behavior of different cystinuric individuals as suggested by Andrews & Randall (86) was also noted. Lewis *et al.* have also offered evidence which indicates that as the level of protein metabolism is increased the ability of the cystinuric organism to utilize normally the sulfur-containing complex which gives rise to cystine in the urine of the cystinuric is also increased.

According to Brown & Lewis (87) the specific rotation of a purified sample of cystine which had crystallized spontaneously from cystinuric urine offered no evidence of differing significantly from the maximal values usually given for the rotation of *L*-cystine from

protein hydrolysates. This work, along with that of Loring & du Vigneaud (88) who found that no differences existed in the individual or mixed solubilities of the cystine isolated from a urinary calculus and the cystine isolated from hair, would seem to dispose completely of the question that has been raised in recent times as to the identity of stone and hair cystine.

The literature on the occurrence of cystinuria in dogs has been reviewed by Green, Morris, Cahill & Brand (89). It was brought out that the condition was apparently recognized in this species a few years after Wollaston's discovery (90) of the human urinary calculus. Evidence is found in this paper to indicate an hereditary nature of canine cystinuria and work has been started in an attempt to re-establish the disease by interbreeding among the descendants of a cystinuric dog. The results will be awaited with much interest.

It has been necessary to forego reviewing certain topics which we hope may be considered by future reviewers. Because of the exigencies of space, we have omitted discussion of the relation of sulfur compounds to certain phases of the chemistry of enzymes, toxins, and hormones, and to such fields as detoxication, fat metabolism, and selenium toxicity.

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CHEMISTRY AND METABOLISM OF THE NUCLEIC ACIDS, PURINES, AND PYRIMIDINES*

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NUCLEIC ACIDS, NUCLEOTIDES, AND NUCLEOSIDES

Final evidence is not yet at hand with respect to the constitution of the nucleic acids; the linkage of nucleotides is still unknown. Makino (1) supposes that the nucleotides in animal as well as in yeast nucleic acids are linked as esters. The assumption rests on the increase of acidity which follows upon a chemical or fermentative hydrolysis of nucleic acids. Klein & Rossi (2) decline to postulate a ring structure, because thymus nucleic acid is easily split off by thymonucleinase.

By boiling yeast nucleic acid in water, Brederick & Richter (3) obtained a substance which they were able to identify as guanine-uridylic acid. Guanylic and uridylic acid, linked by a nitrogen-phosphorus bond, are therefore in a vicinal position in the yeast nucleic acid molecule.

Myrbäck & Jorpes (4), from chemical analysis and the speed of diffusion, ascribe to pancreas nucleic acid a pentanucleotide structure of molecular weight 1645. At any rate they do not believe that pancreas nucleic acid is a mixture of yeast nucleic acid and guanylic acid. Steudel (5) isolated from the pancreas nucleoproteid of Hammarsten a nucleic acid which he calls "allonucleic acid," and which until now he thought was a compound of five or seven simple nucleotides, four of the molecules being guanylic acid and one adenylic acid.

Ducceschi (6), when extracting nucleoproteids from tissues, avoids the disadvantages of the long extraction methods by preparing the tissue with formalin. Feulgen (7) reports a simple method for the preparation of the *b*-thymus nucleic acid from the *a*-structure by means of pancreatin "Merck." The effect is due to a nucleogelase, contained in the pancreatin. He regards this process as a depolymerisation.

Methods for the preparation of adenylic and inosinic acids, used by Embden, Lohmann & Ostern, are fully described by Embden & Schmidt (8). Klein & Thannhauser (9) first succeeded in synthesis-

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ing the two pyrimidine nucleotides in crystalline form. Levene & Tipson (10) report a partial synthesis of muscle inosinic acid: inosine is obtained from adenosine by means of acetic acid and barium nitrite; by phosphorylation with phosphorous oxychloride in pyridine it changes into 5-phospho-2,3-mono-acetoninosine, the barium salt of which is identical with that of muscle inosinic acid.

Newer methods for the determination of adenylic acid have been introduced by Lohmann and by Ferdmann [Embden & Schmidt (8)]. Buell (11) mentions an improved modification of the Buell-Perkins method for the determination of adenine nucleotide in human blood; the normal value is 22 to 37 mg. per cent. By means of this method of Buell & Perkins, Allen and coworkers (12) have proved that the proportion of nucleotide-nitrogen is almost parallel to the number of red blood cells. Buell (13) found similar results with his new method. Whether hemoglobin and adenylic acid influence each other or whether both are regulated by a third factor, has not been decided.

Nucleotides and nucleosides have a certain importance in therapy. In leucopenia, especially agranulocytosis, they are said to stimulate the leucopoietic apparatus. Hoffmann (14), in experiments on rabbits, found that adenylic acid, in contrast to guanylic acid, caused the strongest leucocytosis. The pyrimidine nucleotides were without effect. Jono (15) describes a number of pronounced pharmacological effects produced by cytosylic acid on the rabbit and the frog. Uracilic acid proved to be pharmacologically inactive.

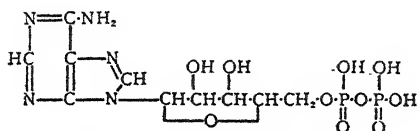
Makino (16) confirms the furanoid structure of desoxynucleosides. Levene & Tipson (17) proved this structure for thymidine also.

Lehmer (18), in the laboratory of Schuler, reports the determination of small quantities of nucleosides in slices of tissue and in serum. The method is based on the ability, discovered by Schuler & Reindel, of the tissue slices of pigeon pancreas to oxidise nucleosides and free purines to uric acid. According to Cerecedo & Allen (19) guanosine and adenosine are perhaps destroyed in different ways in the dog. They assume that urea is obtained from guanosine by way of allantoin.

NUCLEOSIDE-POLYPHOSPHORIC ACIDS

The constitution of adenylypyrophosphoric acid and of adenosinediphosphoric acid has been elucidated by Lohmann (20) by chemical and physical investigations of adenosinediphosphoric acid, which he prepared by a biological method. On the basis of these findings, Loh-

mann proposes the following structure for adenosinediphosphoric acid:



Adenosinediphosphoric acid

In adenylypyrophosphoric acid three molecules of phosphoric acid are mutually esterified. Makino (21) has come to the same conclusion; he also obtained four and six acid valences, respectively, by electrotitration before and after hydrolysis of the polyphosphoric acid. Therefore, the definition suggested by Barrenscheen & Filz (22) has to be abandoned.

Wagner-Jauregg (23), by mixing the aqueous solution of adenosine-mono-, di-, and -triphosphoric acids with alcoholic acridine, obtained salts of the adenosinepolyphosphoric acids that are difficult to dissolve in cold water, more readily soluble in hot.

New methods for the isolation of adenylypyrophosphoric acid and adenosinediphosphoric acid from the muscles of calf heart, are given by Lohmann (24) and Parnas & Lutwak-Mann (25). The biological production of adenosinediphosphoric acid from adenylypyrophosphoric acid by means of washed crab muscle is very interesting.

The assumed existence of a special heart nucleotide (Embden, Ostern, etc.), with the structure of a di-adenosinepentaphosphoric acid is no longer tenable according to the investigations of Lohmann & Schuster (26). Only adenylypyrophosphoric acid was found in the musculature of fresh living frog heart, also in calf heart. The different findings of other authors are explained by the fact that hearts already autolysed were used as sources of the polyphosphoric acids. In the light of these results the findings of Ostern & Baranowski (27), who report an enzymatic preparation of the heart nucleotide, and the observations of Ferdmann & Galperin (28) lose much of their importance. Further details about the preparation and determination of the adenylypyrophosphoric acid are given by Embden & Schmidt (8).

COFERMENT (WARBURG) AND COZYMASE (EULER)

During the last two years interesting and new findings have been made about the chemistry and biology of the fermentation coenzyme.

Warburg & Christian (29) discovered in horse blood cells an adenine-pyridine-nucleotide that proved to be identical with the hydrogen-transporting coenzyme (30). The empirical formula is $C_{21}H_{28}N_7P_3O_{17}$ and it consists of one molecule of adenine, one molecule of the amide of nicotinic acid, three molecules of phosphoric acid, and two molecules of pentose, combined under liberation of six molecules of water. This compound, characterised by Warburg as a hydrogen-transporting enzyme, reacts with hydrogen donators in the presence of special protein substances, so-called *Zwischenfermente*, by absorbing two atoms of hydrogen. The coenzyme, thus reduced, is able to transfer this hydrogen, e.g., to the yellow oxidation ferment. The hydrogen transfer is effected by the pyridine nucleus (pyridine \rightleftharpoons dihydropyridine). The substance is widely distributed in nature and is identical with that of yeast [Warburg, Christian & Griesse (29)]. The Euler school has simultaneously come to almost the same conclusion (31). Karrer, Schwarzenbach, Benz & Solmssen (32), from model experiments on several derivatives of the amide of nicotinic acid, suggest that the nicotinic-acid-amide in the coenzyme is linked as a quaternary salt.

The cozymase of yeast, according to the findings of the Euler school (33), contains two codehydrogenases: the cozymase itself and the coferment of Warburg, called codehydrase II by Euler. Cozymase is, therefore, also designated by Euler as codehydrase I. Euler and coworkers (34) have recently reported the separation and analysis of codehydrase I and II. The results are given in Table I. Das

TABLE I

COMPOSITION OF CODEHYDRASE

	Codehydrase II (Warburg)	Codehydrase I (Euler)
Bases	Adenine; nicotinic-acid-amide	Adenine; nicotinic-acid-amide
Sugar	2 mol. pentose	2 mol. pentose
Phosphoric acid	3 mol. phosphoric acid	2 mol. phosphoric acid, besides Ca-substances
Titration	Tetra- or pentabasic	Dibasic

(35) reports on the distribution of codehydrase II in nature. Adler & Michaelis (36) did not succeed in finding it in heart muscle.

The structure of codehydrase I has not been fully elucidated; on the basis of highly purified zymase, it is regarded by Euler as a

dinucleotide. Thus the ideas of Myrbäck (37), who still characterises the cozymase as a mononucleotide (compare his exhaustive review of literature), are obviously untenable.

The preparation and exhaustive purification of cozymase, based principally upon its isolation as a complex salt of monovalent copper, are reported by Euler, Albers & Schlenk (34). Warburg and co-workers (39) isolated the coferment from the red cells of horse blood in five stages of purification. They declare that the quantity in 1 litre of horse-blood cells amounts to 12 mg. The determination of the coferment, according to the statements of these authors (40), may be made by measuring its hydrogen-absorbing capacity; one mol of coferment absorbs one mol of hydrogen.

As we have seen, the structure of the cozymase is of interest in the chemistry of nucleic acids; its importance, however, is further increased by its relationship to the processes of fermentation and of muscle energetics; it can, indeed, be compared with the so-called adenylic acid system, the coenzyme function of which was discovered by Lohmann. By the works of the Heidelberg, Lemberg, and Stockholm schools this clear participation of the above mentioned coenzymes in muscle and fermentation mechanisms has very rapidly become evident during the last few years and has met with an almost astonishing unanimity of result. It has been proved (41 to 62) that in the processes of fermentation and of muscle contraction, adenylic acid is able to absorb phosphate (acceptor function) from phosphate donators (phosphopyruvic acid, phosphocreatine, and hexosediphosphate); on the other hand adenylypyrophosphoric acid is able to deliver phosphate (donator function) to carbohydrate, creatine, and water (Parnas). All these components being present, we have, therefore, a system of esterification (*Umesterungssystem*), complete in itself. In the same way the cozymase acts as coenzyme in the glycolysis of muscle extract and in fermentation, it being assumed that hexosediphosphate will be present as an induction-compensating factor. According to Euler cozymase shows two activator functions in this process: an oxidoreductive and a phosphate-transferring function. Vestin (62) has recently clearly elucidated these complicated reactions.

It is interesting that Lohmann not only found arginine-phosphoric acid in the muscle of *Octopus*, but also adenylypyrophosphoric acid. The decomposition of arginine-phosphoric acid takes place also by way of the adenylic acid system (64), which therefore plays a rôle in

lactic acid production in this species of animal as well. The muscle, however, acts like mammalian muscle in that it is poisoned by iodoacetic acid. Lohmann (65) states that the muscle adenylic acid desaminase is missing from the muscle of the crayfish; therefore, the formation of ammonia, in contrast to the opinion of Embden, is not a direct cause of muscle contraction.

PURINES AND PYRIMIDINES

Frèrejacque (66) investigated the autoxidation of uric acid in alkaline solutions and in the presence of catalysts; he found a ureide, which he thinks is oxacetylene-diurein-carboxylic acid. Hilbert and coworkers (67) succeeded in partially synthesising cytosine: 2,4-diethoxypyrimidine was converted, by means of alcoholic potassium hydroxide, into 1,2-dihydro-2-keto-ethoxypyrimidine, which, treated with ammonia at 120°, yielded cytosine almost quantitatively. Johnson & Litzinger (68), continuing the work of Donleavy (69), have succeeded in synthesising an aliphatic amino derivative of the uracil series (uracil-5-methylamine). Hunter (70) reports an interesting decomposition of guanine to 4- (or 5-) guanidinoglyoxaline, an intermediate product in the decomposition to glycine.

The high values for uric acid obtained in Grigaut's method for the determination of uric acid have finally been explained by the investigations of Coste & Grigaut (71). They have found that, by their method, sulfur substances and vitamin C react like uric acid. The method, therefore, is unsuitable for unprepared blood. Dmochowski and coworkers (72) offer a micromethod for tissue guanine which leads to remarkably smaller values (7 to 10 mg. per cent) than those found by Schmidt (23 to 27 mg. per cent). Graff & Maculla (73), also, have modified Schmidt's method. Another method for the determination of uric acid in tissue slices may be mentioned, that of Edson & Krebs (74), which cannot be used for blood analysis.

Hunter (75) describes an interesting reaction of thymine with the diazo-reagent in the presence of sodium carbonate, sodium hydroxide, and hydroxylamine. The test is positive in solutions containing less than 0.01 mg. of thymine. It quite definitely distinguishes thymine from uracil or cytosine.

Rangier (76) maintains that uric acid occurs in the urine as a ureide compound together with urochrome. This complex, stable at pH 6.2, accounts for the great solubility of uric acid. Alterations in

pH cause the compound to dissociate and lead to the precipitation of uric acid; in diseases of the kidney this complex-formation and dissociation also take place. The uric acid-urochrome complex is a redox system (77).

The mechanism of uric acid excretion through the kidneys is still unknown and is not cleared up by the extensive experiments of Berglund and coworkers (78), who deny a tubular reabsorption, nor by Gärdstam (79) who, through using quite similar methods, finds an opposite result.

The investigations of Moraczewski and coworkers (80) on the influence of mineral metabolism on uric acid metabolism seem to be of importance. The increase of uric acid in blood and urine after feeding with alkali is interpreted as a retention or as the result of an increased uric acid synthesis from protein. Chrometzka and coworkers (81), after doses of alkali, found in the dog the same intensified excretion of uric acid and oxyuric acid (*X-fraction*) and interpret this fact as evidence of a certain retardation of metabolism by alkali.

Considering the difficulties that exist in defining the influence of hormones upon purine metabolism, the findings of Chaikoff and coworkers (82) are rather important: insulin, by a secondary mobilisation of adrenaline, causes a considerably increased excretion of allantoin or uric acid. Miyahara (83) had previously observed the same effect on the rabbit.

Purine-metabolism experiments, after injury to the liver, have been made by Chrometzka and coworkers (84, 85, 86). In acute inundation of the isolated and perfused dog liver with India ink, as well as *in vivo* after intravenous doses of India ink, considerable retardation of uricolysis takes place. When this damage by ink is continued, the inhibition of the ferment action becomes irreversible. Histological examination of the liver revealed an intense fat-cirrhosis in the most extreme cases. Similar observations were made by Domini (87) after trypan-blue and pyrodine injections and by Daft and coworkers (88) after liver damage with chloroform.

The problem of gout has not yet been solved. Gudzent regards it, in all its clinical manifestations, as an allergic disease. Labbé and coworkers (89) believe that, in the case of gout, uric acid circulates in the blood in a different physical form or as an altered chemical compound. Coste & Grigaut (cf. 89) consider gout to be a disturbance of protein metabolism, the consequence of which is an over-production

of uric acid. According to Weil and coworkers (90) the cause of gout is not hyperuricaemia itself, but the increase of uric acid is evidence of a hyperergic reaction during a crisis in liver metabolism.

Convay & Cerecedo (91), continuing former experiments on man and dog (92), have found that, in the rabbit, the decomposition of isobarbituric acid takes place partly by way of urea; it is excreted partly as ethereal sulfate. In the growing organism, the conditions studied by Cerecedo & Stekol (93) are different. In this case no formation of urea takes place after the feeding of isobarbituric acid; the acid is excreted, for the greater part unchanged, but to a lesser degree in conjugation with sulfuric acid. Perhaps a synthesis to purines may even take place.

SYNTHESIS OF URIC ACID

Continuing their former investigations, Schuler & Reindel (94) suggest that the nitrogen source for purine synthesis is ammonia or amino acids; until now they have not been able to identify the carbon source. Neither nucleotides, nor nucleosides, nor free purines are the primary substances. In contrast to their opinion Edson, Krebs & Model (95) believe that hypoxanthine is the first step in uric acid synthesis in the avian organism. Chrometzka & Gottlebe (96), after disturbing the synthesis of uric acid in the capon by blocking with India ink, have found an increased excretion of amino acids and ammonia. The purine-base fraction was not increased in this suppression of uric acid synthesis. Allantoin has never been found. The observation made by Fisher (97) who, on feeding birds with *l*-lactic acid, found an increased uric acid excretion, but no increase after *d*-lactic acid or other C_3 -substances, is very interesting. The well-known results of Wiener are discarded by the author as being inadequate.

Uric acid synthesis in molluscs has been reported in two remarkable studies (98, 99) which come to the same conclusions. Baldwin (98) found that the liver of *Helix pomatia* builds up uric acid from urea and tartaric acid; in this process uræa is obtained from arginine by means of arginase. This formation of uric acid is a detoxication mechanism ["Excretion-synthesis," Schuler & Reindel (94)]. Aquatic animals produce but little uric acid, because they excrete uræa itself in the water. The amount of uric acid synthesised by terrestrial

animals is proportional to the dryness of their habitat [Needham (99)].

ENZYMES OF NUCLEIC ACID METABOLISM

The nucleogelase (Pancreatin-Merck) of Feulgen (7), which depolymerises polynucleic acids, has been mentioned earlier (cf. page 211). Makino (100) refers to this enzyme as polynucleotidase. Ishikawa & Komita (101) isolated nucleotidase from dog and cat pancreas extracts and from kidney and muscle fluids of rabbit. In the purine ribonucleotides these purified enzyme preparations hydrolysed the glucoside compound which Schmidt (102) suggests is possible only after previous desamination of the adenylic and guanylic acid. Lohmann (103) has prepared pyrophosphatase and has shown that the washed residuum of crayfish muscle splits off only one molecule of phosphoric acid from adenosinetriphosphoric acid—an easy biological method for producing adenosinediphosphoric acid (24).

An evidently practical method for purifying nucleosidase is described by Klein (104): the starting material may be spleen, lungs, liver, or heart muscle; the highest yield is obtained from spleen; purification is effected by repeated adsorption on aluminium-oxide-Cy and elution with 0.05 *M* sodium arsenate (an activator of the nucleosidase). This enzyme, free of phosphorus and containing but 4 per cent of nitrogen, is able to hydrolyse a fifteenfold quantity of guaninedesoxyriboside (best substrate). The optimum pH is 6.5. The preparation is specific for purine nucleosides. The pyrimidine nucleosidase is excluded by this method of preparation. Boyland & Boyland (105) found adeninenucleosidase in tumor tissue (Jensen-Rous sarcoma).

The investigations of Keilin & Hartree (106) concerning uricase and xanthine oxidase are noteworthy: the optimum pH is 9.25; the equation for the uricolytic reaction is $\text{uric acid} + \text{H}_2\text{O} + \text{O}_2 \rightarrow \text{allantoin} + \text{CO}_2 + \text{H}_2\text{O}$; xanthine oxidase is destroyed by catalase.

The old problem, whether the Schardinger enzyme is identical with xanthine oxidase is answered in the affirmative by Booth (107), who determined the time of first appearance of the two enzymes in the yolk sac and whole embryo, the action of inhibiting agents, activity ratio, etc. Andersson (108), however, finds that the cozymase, adenylypyrophosphoric acid, adenylic acid and adenosine do not hinder the dehydrogenation of xanthine by the Schardinger enzyme and

methylene blue; whereas they do inhibit the dehydrogenation of acetaldehyde by the Schardinger enzyme and methylene blue.

NATURAL PIGMENTS WITH A PURINE OR PYRIMIDINE SKELETON

According to the investigations of Wieland & Schöpf (109), and Wieland and coworkers (110), xanthopterine, a pigment from the wings of the brimstone butterfly (*Gonopteric rhamni*) and leucopterine [white pigment of the cabbage butterfly (*Pieris rapae*)] are animal redox pigments which give a positive murexide test. Schöpf & Becker (111) give as the empirical formula for xanthopterine, $C_{19}H_{18}O_6N_{16}$. Nothing is known about its constitution. Koshara (112) recently discovered a substance similar to xanthopterine if not identical with it, in the urine of man. These pigments, according to the experiments of the above-mentioned investigators, are widespread in the animal and vegetable kingdoms and have been detected in man, ox, rabbit, horse, and several plants.

In 1935 a blue-fluorescent pigment of yeast, afterwards called thiochrome, was isolated and its constitution explained by Kuhn and coworkers (113, 114), and at the same time by Barger, Bergel & Todd (115). It contains a pyrimidine group in its molecule. This pigment is interesting because of its relation to vitamin B_1 (Aneurin). Williams and coworkers (116, 117) split vitamin B_1 by means of acid sulphite solution and obtained 4-methylthiazol-5-carboxylic acid as a product of decomposition; a second component, $C_6H_9O_3N_3S$, was assumed to be a pyrimidine derivative. The final determination of its constitution was made in the laboratory of Windaus, especially by Grewe (118, 119). The assumed existence of a pyrimidine ring in the vitamin molecule¹ was confirmed by synthesis, which had been accomplished simultaneously, by a similar method, in the laboratory of I. G. Farbenindustrie by Andersag & Westphal.

Investigations on the constitution of vitamin B_2 (lactoflavin) have become equally interesting in purine metabolism. Kuhn & Weygand (120, 121) succeeded in determining its constitution. According to this work lactoflavin ($C_{17}H_{20}N_4O_6$) is a 9-*d*-ribityl derivative of 6,7-dimethylisoalloxazine. The same investigators were also the first to synthesise a flavin isomer. The vitamin itself was synthesised at the same time by Karrer (122) with the aid of the previous work of

¹ For formulae of vitamin B_1 and thiochrome see pp. 335 and 336 (EDITOR).

Kuhn. The constitutional formula² demonstrates the relation of vitamin B₂ to the purines. Lactoflavin is also used by the animal organism for the formation of the yellow oxidation ferment [Warburg & Christian (123)]. It has been obtained in crystalline form by Theorell (124). The effective group, combined with a protein carrier, is lactoflavin phosphoric acid. A synthesis of the latter has recently been described by Kuhn and coworkers (125).

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² See page 508 (EDITOR).

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CARBOHYDRATE METABOLISM*

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ABSORPTION OF CARBOHYDRATES

Glucose is absorbed by dogs more rapidly than sucrose as demonstrated by a greater increase in blood sugar following the administration of glucose (Roberts). The absorption of galactose and glucose is controlled by different factors than that of xylose (Westenbrink & Middelbeek), in harmony with earlier observations of Verzár and his co-workers. Westenbrink, however, in experiments upon rats, could demonstrate little variation in the rate at which glucose was absorbed, when mixed with phosphate buffers, except a depressing action when the concentrations of buffer were great. According to Chandhuri & Kahali, the glucose absorption in amygalized cats was much less than in rats; it was greatest (48 mg. per 100 gm. body wt.) when given in a 10 per cent solution in doses of 1 gm. per kg.; the rate was slightly depressed by insulin. In the gastro-intestinal tract of man, Abbott, Karr & Miller found that the glucose concentration during absorption was variable; however, it rarely exceeded 5 per cent except near the ligament of Treitz. The ketose, sorbose, can be taken up from the gastro-intestinal tract as readily as glucose or fructose [Grieshaber (1)].

The absorption of glucose was found by Bennett to be much depressed in hypophysectomized rats. Thus, the average glucose assimilation in operated animals for two successive hours was only 141 and 135 mg. per hour per 100 gm. of body weight, compared with control values of 223 and 211 mg. The decrease in rate of absorption reaches a maximum within a short period after the operation according to Samuels & Ball. However, the depressed glycogen formation is not solely the result of a decreased absorption rate as hypophysectomized rats form proportionally less glycogen from definite amounts of glucose. Buell, Anderson & Strauss also show that the rate of absorption of *d*-lactic acid is definitely lower in adrenalectomized than in normal animals.

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BLOOD SUGAR AND CARBOHYDRATE TOLERANCE

One of the most interesting studies during the year of the relationship of blood sugar to carbohydrate metabolism was reported by Wierzuchowski (1). When glucose was injected intravenously in dogs at constant rate in excessively large doses, a condition referred to as "overflow diabetes" occurred. The maximum glucose utilization was found to be 3.09 gm. per kg. per hour at a blood sugar level of 600 to 700 mg., but this rate of utilization was not reached until glucose was given at a rate of 7 gm. per kg. hourly. When the rate of injection of glucose was further increased, the additional sugar was almost completely rejected in the urine (97 per cent)—hence the resemblance to a diabetic animal. Moreover, no increase in lactic acid, heat production, or oxidation could be shown to result from any additional glucose given over the 7 gm. rate. The glucose level of the blood varied from an average of 148 mg. when the dose was 1 gm. per kg. to over 1900 mg. per 100 cc. when 9 gm. were given per kg. per hour. Wierzuchowski (2) noted that the specific dynamic action and oxidation were greatest when the blood sugar varied between 1000 and 2000 mg. At a level of 2500 mg. the dogs were restless, and transitory nystagmus was noted with "marionette-like" movements. Clonic convulsions and opisthotonus were characteristic of levels of 3000 mg. whereas at 3500 mg. violent nystagmus and a state of excitation occurred. This was followed by a depression at a level of 3700 to 3800 mg. just prior to death. If the blood sugar was maintained for several hours at 2000 mg., albuminuria usually developed since the animals were unable to withstand the strain of the excessive glycosuria. If the blood sugar level was raised only transitorily to 2500 or 3000 mg., some animals recovered if the attendant diuresis persisted. Somewhat similar symptoms are reported by Jacobs & Colwell after prolonged injections of lower amounts of glucose (0.7 to 4.5 gm. per kg. per hour) into dogs. There was an early and sustained ability to utilize carbohydrate which failed terminally. Death resulted after a specific and intense hemorrhage into the anterior lobe of the pituitary and the pancreas; this may result from an acidosis caused by the accumulation of an unidentified acidic intermediate.

On the other hand, according to the results of Dotti & Hrubetz, the level of fermentable blood sugar reaches zero in rats, after insulin, when hypoglycemic convulsions occur. During the course of insulin shock there is no appreciable change in the non-fermentable portion.

Glucose tolerance is not regulated, according to Soskin, Mirsky, Zimmerman & Heller, by an increased insulin secretion but rather by a homeostatic mechanism of the liver whereby this organ decreases its supply of glucose to the blood in the presence of exogenous sugar. In hypophysectomized-depancreatized dogs normal glucose-tolerance curves are obtained due to the fact that the antagonistic effects of the pancreas and hypophysis are removed. Glucose tolerance in diabetic children is favorably affected by the administration of large doses of sodium chloride (1 to 2 gm. per kg.) according to McQuarrie, Thompson & Anderson, while potassium salts produce a diametrically opposite effect. As little as one-third of an equivalent of potassium completely antagonizes sodium. Crabtree & Longwell have shown the favorable effect of high sodium chloride diets on glycogen deposition in rats.

Forbes noted no appreciable change in blood sugar in acclimatized subjects at rest or work at altitudes as high as 6140 meters. Aisner, Gorney & Segal state that section of the vagus nerves below the diaphragm did not alter the glucose tolerance in normal dogs, although it was somewhat increased after unilateral adrenalectomy and denervation of the remaining adrenal. Bilateral section of the splanchnic nerves, two to twenty-two days previously, was also found by Sunaba (1) to be without effect on the blood-sugar changes following glucose in rabbits fasted for twenty-four hours. Bodo & Benaglia conclude from experiments on cats that sympathin, produced by electrical stimulation of sympathetic nerves or by emotional excitement, increases blood sugar. The blood-sugar content of monkeys suffering from experimental poliomyelitis was found by Jungeblut & Resnick to be similar to normal animals although the glucose-tolerance test was somewhat prolonged. No variations in blood sugar or in glucose tolerance were found in men or rabbits infected with *Trichinella spiralis* (Augustine) nor in allergic conditions (MacQuiddy, McIntyre & Koser). Hyperglycemia caused by traumatic shock is related to increased glycogenolysis by the liver (Stolfi).

Neuwirth refutes the earlier conclusions of Olmsted (1) in which it is stated that the blood cells of various species (including man) contain no glucose. Although the plasma to cell ratio in human blood to which no oxalate was added was 1.43, it was much higher in rabbit blood which contained only 13.5 per cent as much sugar in the cells as in the plasma. Olmsted's later results (2), in which it is stated that oxalate alters the permeability, are also denied. Britton, Silvette &

Kline report that the ratio of plasma sugar to cell sugar is 1.32 in normal cats although this proportion is increased by adrenalectomy. Knapp notes that the arterial blood sugar is always higher than the venous blood sugar in the case of cattle and chickens. The average difference in the case of cattle was 8.5 mg. per 100 cc. of blood while the mean level of the arterial blood sugar of chickens was greater by 17 mg. per 100 cc. of blood than that of the venous blood sugar.

Blood sugar is increased by carbon monoxide poisoning in mice to the point of glycosuria due to the stimulating action of carbon monoxide on the adrenals and the thyroid (Schulze). Ergotamine hydrochloride lowers the blood sugar in rabbits and prevents a hyperglycemia after adrenaline (Lipschitz) although it possesses no influence on glycogenolysis in the muscle in the presence or absence of adrenaline (Overbeck). Absinthe usually increases blood sugar in rabbits according to Hrubetz & Pike while Hrubetz (2) reports that physostigmine causes a sharp rise in blood sugar—an increase, however, which can be completely prevented by atropine. The latter drug also prevents the hyperglycemic action of pilocarpine [Hrubetz (1)]. Acetylcholine is another drug which produces hyperglycemia, according to the same investigator, through a direct effect on the vagus. Phosphotungstic acid, phosphomolybdic acid and many allied substances were found by Mukherjee to cause a hypoglycemia in rabbits when administered in a dose of 0.3 gm. These act as oxidases while phosphotungstic acid is also a diuretic. They also showed some activity when introduced into diabetic patients.

The blood sugar can be maintained at a more uniform level in diabetic subjects by obtaining carbohydrate from protein (Conn & Newburgh). Less is lost by glycosuria because of the slow rate of formation of sugar with its consequent slight effect on blood sugar. Similarly, this source of carbohydrate is more satisfactory in cases of spontaneous hypoglycemia due to hyperinsulinism (Conn); the blood sugar is not rapidly increased so no additional stimulation to insulin production occurs. Macallum suggests the possibility of treating hyperinsulinism with an inhibiting factor prepared from the duodenum. This principle is thermostable and its effect may persist as long as sixty days. It exercises a hyperglycemic effect. Gray reports favorable clinical results on the insulin tannate of Maxwell & Bischoff; it is found to be 20 to 25 per cent more effective than insulin and its effect is considerably prolonged. Similar differences are clearly dem-

onstrated on rabbits by Bischoff (1). Thymus histone has also been used satisfactorily by this investigator (2) to retard the action of insulin and other hormones. When given intravenously, its action approximates that of insulin. On the other hand, when given in massive doses intramuscularly, a prolonged hypoglycemic response was noted without any evidence of insulin shock. Scott & Fisher note that the addition of zinc to insulin, prior to mixing with protamine, prolongs the action of the insulin—indicating the possibility that zinc or other metals may play a rôle in the combination of insulin and protamine.

SOURCES OF CARBOHYDRATE

Although it is generally agreed that 58 per cent of meat protein is convertible to sugar in the diabetic dog, the evidence for the transformations of the individual amino acids in the normal animals is quite fragmentary. Butts, Dunn & Hallman have studied this problem on normal rats to ascertain the glycogenic action of amino acids as well as their ketolytic behavior. Glycine was shown to be glycogenic and also to exert a ketolytic effect when administered to fasting rats having an artificial ketonuria produced by the administration of sodium acetoacetate by stomach tube. On the other hand the administration of *dl*-alanine was followed by a significantly higher glycogen formation and a greater antiketogenic effect despite the fact that both glycine and alanine are considered to be quantitatively convertible to glucose in the phlorhizinized dog. Moreover, *d*-alanine was demonstrated to be about twice as good as a glycogen former and superior ketolytically to racemic alanine. This indicates that only *d*-alanine is effective while the *l* isomer is probably entirely inactive. The glycogenic and ketolytic activities of the two isomers are similar to those exhibited by the related lactic acids (Shapiro).

More recently Butts, Dunn & Blunden have found that *d*-glutamic, *dl*-glutamic, *l*-aspartic, and *dl*-aspartic acids are good glycogenic and ketolytic agents. Although glycogen formation could not be demonstrated after single doses, the rats were able to tolerate small amounts of the acids given hourly by stomach tube, and glycogen levels as high as 3.00 per cent were found in the rats fed aspartic acid over a twelve-hour period compared with controls of 0.40 per cent. After *dl*-aspartic acid lower values for liver glycogen were consistently found than were obtained after the natural isomer, which would seem

to throw doubt on the glycogenic action of *d*-aspartic acid. After the continuous feeding of *dl*-glutamic acid, levels of glycogen well over 1 per cent were noted. Similarly, it was found that these acids were satisfactory ketolytic agents as demonstrated on the artificial ketonuria produced by the procedure of Butts & Deuel. Reid (1) has been unable to show that glycogen formation proceeds in the livers of cats under chloralose anesthesia when propionic, glutamic, or aspartic acids were injected, although positive results were noted when glucose, lactic acid, glycerol, or alanine were introduced. Glycine caused a decided fall in liver glycogen.

The possibility that fatty acids may be precursors of glucose is revived by Young. On the basis of work of Wierzuchowski & Fiszal he calculates that the liver of a 10 kg. dog produces about 60 gm. of glucose per day. Such a value (0.25 gm. per kg. per hour) is also that given by Mann as the amount required to keep the blood sugar of a hepatectomized dog constant. Since this amount of sugar also is set free after a prolonged fasting period it must originate from non-carbohydrate sources. At least 40 per cent can not be accounted for after assuming that maximal quantities originate from protein, lactic acid, and the glycerol of the fat. Similar results obtain for depancreatized dogs. The reason that the feeding of fats does not yield additional carbohydrate is that the transformation to carbohydrate is already proceeding at a maximal rate and the extra fat is unable to stimulate additional carbohydrate formation. Drury, Bergman & Greeley conclude from their work on phlorhizinized, hepatectomized dogs that more sugar is normally found in the phlorhizinized animal than can be accounted for by the nitrogen excretion and the glycerol of the metabolized fat. The supposition is that it must originate from fat although these authors do not so postulate. As cogent as such calculations appear, one must admit that such evidence for the transformation of fatty acids to sugar is only circumstantial.

Moreover, Bachrach, Bradley & Ivy refute the earlier work of Chaikoff & Weber by demonstrating that the extra sugar produced after the administration of adrenaline to depancreatized dogs can be accounted for without assuming a conversion of fatty acids to carbohydrate. Also Butts, Blunden, Goodwin & Deuel were unable to demonstrate any glycogen formation in the livers of rats when the ethyl esters of the fatty acids with an even number of carbon atoms (butyric to myristic and oleic) were fed, although appreciable quantities of glycogen were noted when the esters of the fatty acids with

an odd number of carbon atoms (propionic to undecylic) were given. Although no glycogen originated in the livers of fasting rats when natural fats were fed containing predominantly triglycerides of the fatty acids with fourteen or more carbon atoms, Deuel, Butts, Blunden, Cutler & Knott noted a significant deposition of glycogen following the administration of triacetin, tributyrin, tricaproin, and tri-caprylin. Since these fats cannot be stored as such, it is suggested that under such conditions the glycerol becomes available for glycogen synthesis. No greater amounts of glycogen originated than could be ascribed to the glycerol content of the fats fed. Coconut oil, which is made up of a considerable proportion of shorter chain triglycerides, also showed slight but significant glycogen formation. The triglycerides (tripropionin to triheptylin), which contain fatty acids with an odd number of carbon atoms, were also demonstrated to be excellent glycogenic agents. It should be noted, however, that no rise in blood sugar was observed by Kneip in children after glycerol in doses of 0.5 to 0.6 gm. per kg., nor by Behrens, after 30 gm. amounts. Likewise, no increase was noted by the former author when 50 to 75 gm. of olive oil were given.

VARIOUS SUGARS¹

Griffith & Waters report that fructose can be utilized after hepatectomy and that this sugar prolongs the life of eviscerated animals. The ketose must be utilized as such by the muscles and the brain rather than following its transformation to glucose. Davidson, Kermack, Mowat & Stewart, on the other hand, have shown that fructose stimulates the production of insulin by the pancreas but that this hormone has no effect on its removal from the blood. The metabolism of fructose involves two stages: (a) its conversion, independently of insulin, to an unknown substance (probably glucose); (b) the disposal of the latter, aided by insulin. Clark & Murlin have found that fructose has a superior ketolytic action to glucose on the ketonuria induced in dogs by a high fat diet, confirming the earlier observations of Deuel, Gulick & Butts on human subjects. It is difficult to postulate why fructose should possess a superior ketolytic action to glucose if it did not have an independent metabolism rather than acting merely

¹ A recent review by the author on the intermediary metabolism of fructose and galactose is found in *Physiol. Rev.*, 16, 173 (1936).

after transformation to glucose. After pancreatectomy no ketolytic action was noted by Clark & Murlin from fructose. In phlorhizinized dogs Murlin & Manly found that fructose had the greatest protein-sparing action, followed in order by sucrose and glucose. However the ketonuria under such conditions was lowered equally by the three sugars. Grieshaber (1) states that another ketose sugar, sorbose, is capable of metabolism in both normal and diabetic persons. Much of the absorbed sugar is utilized but approximately 12 per cent is excreted, partly as sorbose and partly as a dextrorotatory fermentable sugar (glucose?). That it is utilized also seems evident from the fact that it lowers ketonuria [Grieshaber (2)]. In two doses of 10 gm. each or up to three doses of 20 gm. each the acetone excretion was decreased to as much as one-third of the original level. Its ketolytic activity is equal to that of glucose and superior to the alcohol, sorbitol.

That galactose likewise possesses a metabolism independent of glucose also seems definite. The superiority of this hexose in ketolytic activity over all the common hexoses, first noted by Deuel, Gulick & Butts on fasting human subjects, is confirmed by Clark & Murlin on the ketonuria of dogs produced by a previous high-fat diet, while in depancreatized dogs it possesses an activity approximately equal to that of glucose. It is possible that the superiority of galactose may be traced to the fact that it produces a glycogen less readily broken down (and hence longer retained) than that from glucose, as suggested by Deuel, MacKay, Jewel, Gulick & Grunewald. That such an explanation is cogent now seems evident from the experiments of Bell who proves that the glycogen formed in rabbits after galactose has 18 hexose units compared with one of 12 formed after glucose. In harmony with Jewel and also Harding, Grant & Glaister it is noted that these units are composed of glucose in both cases.

According to Day the cataracts in rats on a diet containing 60 per cent of galactose is related to the persistent high blood-sugar level. Cataracts developed as early as the eleventh day in galactose-fed rats in which the blood sugar averaged 372 mg. per 100 cc. of blood. The mean for the lactose rats was 160 mg. while that for those on glucose, starch, and sucrose, which did not cause cataracts, was about 120 mg. The cataract differs from that noted in vitamin-G deficiency. Carpenter shows, by making simultaneous observations of the alveolar carbon dioxide tension and the respiratory quotient in human subjects after 25 or 50 gm. of galactose, that the rapid rise in the respiratory quotient is at least partly due to the formation of acids in the intermediary

metabolism of galactose. The possibility of the metabolism of fructose and galactose being controlled by a humoral mechanism is indicated in the work of Kansai who obtained an alcohol-ether extract from the deproteinized sera of nephrectomized rabbits which increased the rate of disappearance of these hexoses from the blood stream.

Sucrose was found by Power & Keith to appear in thoracic lymph very rapidly after its intravenous injection into dogs and later to accumulate in considerable quantity in all tissues, especially in the liver and kidney. After injection into men in doses of 0.7 to 1.0 gm. per kg., it was practically quantitatively excreted in the urine; however, only 70 to 80 per cent could be recovered from the urine of dogs after a similar dosage although considerable amounts of fructose also were found in the urine. This would seem to indicate the presence of sucrase in the tissues or blood of dogs.

Blatherwick, Bradshaw, Cullimore, Ewing, Larson & Sawyer have shown that *d*-xylose does not influence the carbohydrate metabolism of rats. Although the nonfermentable reducing substances in the liver, muscles, kidneys, and blood were increased temporarily, no alteration in the glycogen or lactic acid content of the liver and muscles was observed; the blood glucose, however, was significantly increased. More remarkable is the report of Roe & Hudson who showed that *d*-mannoheptulose, a ketose heptose from avocados, caused a rise in the fermentable sugar content of the blood. Its change to glucose is too slow to permit it to act as an antidote for insulin convulsions although if the administration of the heptose was sufficiently early the animals were protected. After oral or intraperitoneal injection some of the unchanged sugar was excreted.

GLYCOGEN

Sunaba (1) found that liver glycogen was increased most by glucose, less by fructose and galactose, and least by mannose. Sucrose and maltose also were found (2) to be glycogenic but lactose was not appreciably so. Essentially similar results are reported by Otomo who also finds that glycogen formation results after the intravenous injection of sucrose. Xylose was without effect on liver or muscle glycogen (Blatherwick, Bradshaw, Cullimore, Ewing, Larson & Sawyer). Stöhr found that methyl glyoxal (1) and ketol (2) gave glycogen, but not after insulin. Liver slices also are able to transform carbohydrate precursors into glucose. Cori & Shine found that the

following rates of conversion obtained, considering that of fructose as 100: dihydroxyacetone 71, glyceric aldehyde 58, α -glycerophosphate 56, β -glycerophosphate 31, glycerol 30, galactose 20, and mannose 9. Anaërobiosis or cyanide poisoning prevented such transformations from taking place.

The glycogen content of the muscles of rats, fasted twenty-four hours, is not influenced by the carbohydrate content of the previous diet (Blatherwick, Bradshaw & Sawyer). Noll & Becker found that the red muscles of rabbits and hens contained only two-thirds to four-fifths of the quantity of glycogen present in the white muscles; the values show much less discrepancy than those reported earlier by Silvette & Britton (1) (0.89 per cent for white and 0.24 per cent for red muscle). However, there is considerable variation in the glycogen values of different red muscles in the chicken.

Lajos believes that vitamin B₁ has a glycogenic activity similar to but lower than insulin. The association of vitamin B₁ with carbohydrate metabolism is also suggested by Peters, who believes this principle is concerned with the oxidation of such degradation products of glucose as lactic and pyruvic acids while its relation to the oxidation of ketone bodies is suggested by Krebs.

Glycogen storage in the fetus takes place largely in the lungs while the liver is principally occupied with hematopoiesis. The latter organ only takes over this function when its blood-forming functions are lessened (Szendi). In the chick embryo Guelin-Schedrina has demonstrated that glycogen deposition in the liver precedes insulin production since it is first noted on the eighth day while there is no evidence of the islands of Langerhans before the ninth day. Reid (2) notes that the nitrogen-sparing action of glucose is lost in pancreatectomized dogs before their ability to form glycogen is abolished. Ellis & Calvin show that glycogen may be retained during much longer periods of starvation in the mussel than in the higher animals. The maximum values of 25 to 60 per cent in the hepato-pancreas fell slowly during inanition but many individuals maintained glycogen levels in excess of 30 per cent for as long as 338 days. Using a new method for the determination of glycogen in the brain, which excludes the reducing galactose of the cerebrosides, Kerr finds that rat brain has 70 to 130 mg. of glycogen per 100 gm. of tissue. Similar values were noted in the brains of dogs (98 mg.) and rabbits (82 mg.) by Kerr & Ghantus. The latter authors further note that this level of glycogen is not affected by fasting, overfeeding, glucose infusion with

or without insulin, nor by phlorhizin followed by adrenaline or pancreatectomy, although overdosage with insulin will markedly lower the glycogen in the brain. The free sugar in the brain was much more labile. The values in dogs varied between 35 and 75 mg. per 100 gm. while in rabbits the limits were between 45 and 86 mg. When the blood sugar was lowered by phlorhizin or insulin, a corresponding drop occurred in the free sugar of the brain. A similar correspondence between the free sugar of the brain and blood sugar was noted in the hyperglycemia following pancreatectomy or after glucose administration. The level was constantly lower than that of the blood except in extreme insulin hypoglycemia. The disturbances in the central nervous system in hypoglycemic shock may be due to a carbohydrate deficit which results both from an insufficient sugar supply from the blood and because of increased utilization by the brain. The sugar of the brain, normally, is at about the same level as that of another essential organ—i.e., heart muscle, and much higher than the sugar in skeletal muscle.

Evans & Bowie report that cardiac glycogen is raised in depancreatized cats and is well maintained in fasting phlorhizinized rats with or without the injection of adrenaline. These authors suggest that heart glycogen is under separate control from skeletal glycogen or at least that an additional unrecognized factor assists in the regulation of this polysaccharide. Himwich, Goldforb & Fazikas also note a positive balance in glucose and lactic acid in the hearts of normal as well as of depancreatized dogs. The respiratory quotient of the diabetic cardiac tissue indicated that non-fatty substances (including lactic acid) were being oxidized. Further discussion of the physiological control of liver glycogen by the endocrine glands is to be found in the following section.

Increasing reports about blood glycogen are found in the literature. Unshelm believes that this polysaccharide is largely confined to the leucocytes, and any increase after carbohydrate may be ascribed to a leucocytosis. He states that the mean value in the whole blood of man is 3 to 5.6 mg. per cent while Shvartz & Pokrovskaya (1) give a value of 35 mg. per cent. According to the latter authors it is increased by oral administration of sucrose; it is 45 per cent higher in acute and chronic liver diseases (2) and in diabetes (3). No parallelism exists between the glucose-tolerance curve and blood glycogen.

Hodgson showed that glycogen was converted quantitatively to glucose by the liver glycogenase of rabbits and that this enzyme like-

wise possessed the activity of maltase. The glycogenase was active only on the alkaline side of neutrality which is contrary to the theory of Macleod that glycogenolysis occurs because acidity in the hepatic cells renders the glycogenase active. The enzyme also required a coenzyme as dialysis inactivated it and the activity was not restored by sodium chloride. No decrease in the glycogenase was noted after convulsive doses of insulin. Vollmar & Koehler report increases of 39 and 35 per cent in the liver glycogenase of fasting and non-fasting rats, respectively, caused by insulin.

THE PITUITARY

The pituitary gland exercises profound influence on the deposition or retention of liver glycogen. Thus, it has been shown by Russell that the glycogen level is much lower in hypophysectomized rats thirty-six hours after the feeding of starch than in normal rats. Bennett reports that when glucose is fed only 5.9 mg. of new liver glycogen per 100 gm. of body weight (or 2.4 per cent of the absorbed glucose) is formed in hypophysectomized rats compared with 60 mg., or 14.5 per cent of that absorbed, in normal controls. The slower rate of formation of liver glycogen is reflected in the progressively decreasing glucose tolerance after hypophysectomy reported by Samuels & Ball. The abnormal fall in the body stores of carbohydrate of hypophysectomized rats during fasting can be prevented by the administration of extracts of the anterior pituitary; the muscle glycogen of fasted rats is raised to supernormal levels following such treatment (Russell & Bennett). The lower carbohydrate level is to be ascribed partly to a higher oxidation of carbohydrate in the hypophysectomized animals; the carbohydrate stores can be maintained for a longer period and carbohydrate metabolism depressed by extracts of the anterior pituitary (Fisher, Russell & Cori). It is concluded that the pituitary normally exercises an anticarbohydrate-oxidizing activity. This higher rate of oxidation of carbohydrate after hypophysectomy also occurs in spite of a lower oxygen consumption (Fisher & Pencharz). Although the storage of glycogen is very low in depancreatized dogs, as it is in hypophysectomized ones, Chaikoff, Gibbs, Holtom & Reichert found considerable stores of liver glycogen in depancreatized hypophysectomized dogs (2 per cent after three and one-half months). Bachman & Toby show that hypophysectomized rabbits have a normal hyperglycemic response to adrenaline

provided glycogen is still in the liver. Any impairment in such response is due to a low liver glycogen and to a relative fixation of muscle glycogen.

ADRENAL CORTEX

The adrenal cortex is also of prime importance in the regulation of the level of glycogen in the tissues. However, the minimum values for the glycogen reserves in adrenalectomized animals can not be traced to the more rapid oxidation of this foodstuff which occurs after hypophysectomy. Rather is it to be ascribed largely to a failure of the adrenalectomized animal to synthesize carbohydrate from the endogenous sources available in normal animals. Evans (1) noted earlier that fasting adrenalectomized rats failed to respond to lowered atmospheric pressure with increased glycogen stores as occurred in unoperated animals. In more recent work (2) the cause of such inability to synthesize additional carbohydrate is traced to the lack of a sufficient supply of this foodstuff from endogenous protein metabolism. Whereas the normal fasting rat responded on being subjected to one-half atmosphere of pressure by a large increase in protein metabolism (as indicated by urinary nitrogen), no such augmentation was noted in the adrenalectomized rats. The sugar and nitrogen excretion were likewise profoundly reduced in adrenalectomized rats after phlorhizin as contrasted with normal animals. Such differences were noted when the adrenal glands were completely removed but not after extirpation of the medullae alone. Evans was unable to restore the intermediary protein metabolism of adrenalectomized rats to normal by extracts of the adrenal cortex. Similar reduction in the glucose and nitrogen excretion following pancreatectomy in cats was noted by Long & Lukens when the adrenal glands were extirpated. Not only does the glycogen synthesis not proceed normally from the protein sources in this condition but even its formation from lactic acid is upset. Buell, Anderson & Strauss have reported that the rate of synthesis of liver glycogen by rats from *D*-lactic acid is much reduced after removal of the adrenal glands although when the charcoal adsorbate of Grollman, Firor & Grollman was given, the animals were able to make excellent use of ingested *D*-lactic acid. Samuels, Butts, Schott & Ball also have noted that *D*-alanine only slightly increases the blood sugar and glycogen stores in adrenalectomized animals as contrasted with its powerful effect in normal animals. In

continuation of their studies on adrenalectomy, Silvette & Britton found that carbohydrate depletion also occurred in the livers and muscles of adrenalectomized opossums and marmots. These animals behave similarly to rats, dogs, cats, and guinea pigs. The low glycogen levels were not to be ascribed to inanition inasmuch as the glycogen loss is much greater than after prolonged fasting. These authors conclude that a direct regulation of carbohydrate is exercised by the cortico-adrenal hormone which affects sodium-chloride metabolism only indirectly. The possibility of an interrelationship between the metabolism of sodium chloride and glycogen is indicated in the experiments of Crabtree & Longwell who found significantly higher glycogen levels in the livers of male rats on a high-salt diet as contrasted with those on the low-chloride diet. No significant alterations in muscle glycogen were noted in the animals receiving the different dietary regimens.

On the other hand, Parkins, Hays & Swingle deny that a direct relationship exists between the adrenal cortex and carbohydrate metabolism. In adrenalectomized dogs deprived of cortical hormone there are no significant deviations of blood glucose from the normal while injections of large amounts of this hormone are ineffective in altering blood glucose in healthy vigorous adrenalectomized dogs or in animals prostrate from the operation. Although some dogs in which the adrenals have been removed at a single stage operation do show decreased blood glucose when in collapse, such blood-sugar changes are inconstant and highly variable. Lastly it is demonstrated that the adrenalectomized bitch, in oestrus (pseudo-pregnancy), maintains herself in normal health for forty to sixty days without the cortical hormone; there is a slight hyperglycemia.

The glucose distribution between blood corpuscles and plasma also is altered when the adrenal glands are extirpated. Britton, Silvette & Kline noted a considerable increase in the proportion of glucose present in the plasma as compared with that in the cells in animals showing symptoms of adrenal insufficiency.

Long & Lukens report that the alleviating effect on pancreatic diabetes, caused by hypophysectomy in the dog, also occurs in the cat; also it was found that a similar amelioration in the severity of the diabetes follows adrenalectomy. The survival time of such animals was eighteen days whereas that of the controls in which pancreatectomy alone was carried out was only four days. Such a protective action is not to be attributed to adrenaline since demedullation

of adrenals or denervation of these glands was not effective. There is no increase in carbohydrate tolerance either by hypophysectomy or by adrenalectomy; the protective effect in the latter case is to be traced to the decreased production of glucose and acetone bodies.

SEX GLANDS

Although the action of such endocrines as the pancreas, hypophysis, and adrenals on carbohydrate is striking, sex likewise plays an important part in controlling carbohydrate and fat metabolism; the action is definite even if less spectacular. The sex differences in the levels of liver glycogen in fasted rats, reported earlier by Deuel, Gulick, Grunewald & Cutler, are confirmed by Blatherwick, Bradshaw, Cullimore, Ewing, Larson & Sawyer. Although Deuel *et al.* were unable to demonstrate any sex difference in the levels of liver glycogen in fed rats, maintained on the usual stock diet, significant differences have since been noted (Deuel, Hallman & Murray) in such values for non-fasting rats partaking of a high carbohydrate-rich butter-fat diet (3.62 per cent for males and 1.93 per cent for females). Similar results have been noted for diets composed of other fats and likewise in rats on the stock diet [4.28 per cent for males and 2.29 per cent for females (Deuel, Murray & Hallman)]. The reason for the earlier failure to note such differences in fed animals may possibly be ascribed to the season of the year, age, a slight change in the diet, or to some factor not clearly recognized. As noted earlier (Gulick, Samuels & Deuel), the sex difference in glycogen levels is abolished by ovariectomy. It is of interest to call attention to the fact that such sex differences have also been described in dioecious plants (Ruhland & Wolf). One wonders if such variations are to be assigned to theelin-like compounds known to exist in the plant kingdom. However, the present author was unable to prove that theelin was the factor which caused the alteration in carbohydrate metabolism in the rat.

The sex variability in carbohydrate metabolism is also reflected in the differences noted in ketonuria. The level of ketonuria is higher during fasting in women than in men (Deuel & Gulick) although no differences occur in the slight ketonuria of fasting rats. After the production of an artificial ketonuria by administration of sodium acetoacetate, sex differences similar to that noted in man are obtained in rats (Butts & Deuel); the differences are abolished by ovariectomy

(Grunewald, Cutler & Deuel). Deuel, Hallman & Murray have recently shown that such sex variation in acetonuria is exhibited by rats which are merely fasted following a dietary regime through which large quantities of fat are deposited in the liver.

Sex differences in carbohydrate metabolism were also found by Dotti in rabbits, who noted that the does were more sensitive to insulin injections than the bucks. Such differences were found both in the initial response to insulin as well as to subsequent responses to repeated insulin injections. These differences obviously are to be traced to variations in their stores of carbohydrate.

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FAT METABOLISM*

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DIGESTION AND ABSORPTION

Normal adult animals appear to be able to absorb quite large amounts of all the fats commonly eaten as foods. However, since the physical properties, such as melting point, of the natural fatty acids cover such a wide range, it has generally been thought that differences exist in the rates of absorption of the various fatty acids from the intestinal tract. The fact that the fecal fatty acids are ordinarily more saturated than those of the food seemed to lend support to that opinion. It is now evident that the fecal lipids may consist largely of fatty material secreted into the bowel rather than unabsorbed food lipids. On exclusion of bile from the intestine of a human subject, the fatty acids in the feces were found to equal or exceed the intake, and yet 65 to 70 per cent of the labeled ingested fat was absorbed (1).

Recent studies have revealed rather striking differences in the comparative rates of absorption of various fats by the rat. For instance, with a fixed dose of 1.5 cc. of fat per rat, the percentage absorption in four hours ranged from about 65 per cent for linseed oil and olive oil, 58 per cent for soy bean and peanut oils, 47 per cent for coconut oil and coco butter, to about 36 per cent for palm oil and oleo stock (2). If given sufficient time, all were completely absorbed. The findings of others suggest that the rate of absorption of fat is governed more by the rate of passage out of the stomach and the rate of enzymic hydrolysis (3, 4) than by differences in the rate of passage of the various fatty acids from the lumen of the intestine into the epithelial cells of the villi.

According to the present-day belief, the fatty acids liberated by enzymic action in the small intestine are absorbed into the epithelial cells and there resynthesized to neutral fat. By means of staining reactions, it has been shown (5) that fatty acids can be detected in the epithelial cells at the tips of the villi within ten to twenty minutes after feeding the fat. By the sixth hour, in the normal animal (rat),

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the epithelial cells are completely filled with fatty droplets which, since they stain with Sudan III but not with the fatty acid stain, appear to be entirely neutral fat.

If the rat be poisoned with iodoacetic acid or phlorhizin, the histological picture is quite different. By the sixth hour most of the epithelial cells contain considerably less fatty material than in the normal rat and this material gives an intense reaction for free fatty acid. The same is found in adrenalectomized rats (6). If a cortical extract be given to the latter an hour or so before the olive oil, then the histological picture is quite normal.

The absence of more than traces of free fatty acid in the epithelium of the normal rat and its accumulation in the poisoned rat appear to fit in well with the chemical evidence that iodoacetic acid, phlorhizin and lack of cortical hormone interfere with fat absorption by preventing the formation of phospholipid, which has been proposed as the first step in the resynthesis of neutral fat (7, 8). The results of more recent experiments involving the use of iodized fatty acids (9) and elaidic acid (10) leave no doubt that ingested fatty acids do become incorporated into the phospholipids of the intestinal mucosa.

When free fatty acids are fed, neutral fat is synthesized in the intestine and appears in the chyle. Apparently, pancreatic diabetes does not interfere with the formation of the necessary glycerol since the chyle of depancreatized dogs contained as much lipid glycerol as did that of normal dogs (11).

There has been, in the past, some evidence to indicate that ingested fatty acids are modified during their passage through the intestinal wall in such a way as to make them approach in character the fat which is normally found in the fat stores of the animal in question. If a fat of low iodine number be fed, the fat in the chyle has a higher iodine number and *vice versa*. Further evidence of the same nature has been published this past year (12); the fatty acids of the chyle of dogs fed horse fat (I.No. 49) had an iodine number of between 62 and 99, with an average of 74. This suggests considerable desaturation of the ingested fatty acids. Furthermore, there was evidence of some oxidation having taken place with resultant shortening of the fatty acid chain, the mean molecular weight of the chyle fatty acids being 260 to 275 as compared with 283 for the fatty acids of the horse fat. These experiments are, of course, open to the same criticism which has been raised against the older ones, namely, that the

changes in the chyle fat may have been due to differential absorption of the various fatty acids in the ingested fat, and admixture of the fatty acids in the intestinal secretions and thoracic lymph. However, neither the amount, nor the mean molecular weight, nor the iodine number of the fatty acids in the control samples of lymph was such as to explain the differences between chyle fat and ingested fat.

Within the past year, what appears to be decisive proof of the desaturation of ingested fatty acids in the animal body has been presented (13). Mice were fed, for several days, fully saturated fatty acids having a high content of deuterium (11 atoms per cent). The purified unsaturated fatty acids of the stored fat were found to have a deuterium content of 1.6 atoms per cent. Since the body water had a deuterium content of only 0.21 atom per cent and since the deuterium content of synthesized fatty acids is much below that of the body water (14), it seems that the unsaturated fatty acids in these mice must have been formed by desaturation of the ingested stearic acid. Where this desaturation takes place has not been determined. It may be that part, at least, occurs in the intestine during absorption.

FAT TRANSPORT: BLOOD LIPIDS

It is a well-established fact that the greater part of the fat absorbed from the intestinal tract enters the blood by way of the thoracic duct. Whether any of the ingested fatty acids enter directly into the portal circulation is still a debated question. In any case, during fat absorption there is in most animals an increase in the amount of neutral fat and sometimes of phospholipid in the general circulation. It appears likely that the immediate destination of most of the absorbed neutral fat is into the fat depots to be stored until later in the day when it is called back into the circulation to supply the necessary fuel for the body.

The increase in phospholipid in the blood plasma has been regarded as evidence that phospholipid serves as a means of transporting fatty acids from the blood into the tissues. What appears to be decisive proof that plasma phospholipid is a transport agent has been arrived at by feeding elaidic acid (10). Within a few hours after its ingestion, this distinctive fatty acid is found to make up about 30 per cent of the fatty acids in the plasma phospholipids even if the latter do not increase in amount. Since the phospholipids of the red cells contain no more than traces of elaidic acid, if any at all, there is no evidence that red cells function in fatty acid transport. Similarly, the

feeding of fats of widely different degrees of unsaturation produces parallel though relatively small changes in the iodine number of the plasma-phospholipid fatty acids (10, 15).

While it is clear that some of the phospholipids in the blood plasma serve as transport agents, there are many reasons for believing that there are others present which do not. Quite recently, evidence has appeared which indicates that 40 to 70 per cent of the phospholipids of plasma consist of sphingomyelins (16). Their function appears to be entirely unknown but there is no reason at present for thinking that they participate with the lecithins and cephalins in fatty acid transport. Methods now being available for determining separately the sphingomyelins, lecithins, and cephalins, it will be possible to determine the type of phospholipid which is involved in the increases and decreases which occur in various physiological and pathological conditions.

The increase in plasma phospholipid during alimentary hyperlipemia, that which occurs during pregnancy in humans and in guinea pigs (17), and possibly that which sometimes but not always (18) occurs in human diabetes, are presumably due to increased transport phospholipid consequent upon the increased fat metabolism. The increase in plasma phospholipid which occurs in other pathological conditions cannot be so reasonably explained. For instance, it has been shown (19) that daily application of suction to the ears of rabbits will cause a severe hyperlipemia involving all of the blood lipids. This hyperlipemia seems to be due to a faulty assimilation of absorbed lipids, but the nature of the derangement is quite obscure. Equally perplexing is the pronounced increase in the phospholipid and free cholesterol of the plasma of rats after ligation of the bile duct (20). The linear relationship between free cholesterol and phospholipid is very striking. It is, of course, generally observed that cholesterol and phospholipid in the plasma vary in the same direction, indicating a functional relationship between the two. Recently it has been found that, in human subjects, inanition tends to cause a drop in both cholesterol and phospholipid in plasma (21).

A new method of determining free cholesterol has been proposed which suggests that the free cholesterol of blood plasma as determined by the usual digitonin procedure consists of an "unbound" and a loosely "bound" fraction (22). Normal human red blood cells contain only "unbound" free cholesterol (22) and no cholesterol esters (23).

Among the diseases involving as one of their symptoms a pronounced change in the blood lipids are: eclampsia, which is characterized by a high phospholipid and low cholesterol and especially a high value for the ratio, [phospholipid]/[cholesterol ester] (24); chronic hemorrhagic nephritis, in which the hyperlipemia occurs only in the chronic active stage and falls in the terminal stage (25); and nephrosis, in which it has been shown that the hyperlipemia cannot be reduced by feeding a low fat diet (26). No satisfactory explanation for the hyperlipemia in these conditions has yet been advanced.

As mentioned above (20), the free cholesterol increases considerably (from 23 to as high as 300 mg. per cent) after ligation of the bile duct. The esterified cholesterol, on the other hand, remains normal. After removal of about two-thirds to three-fourths of the liver, the free cholesterol remained constant on the first day while the esterified cholesterol decreased to about one-half the normal value; then the free cholesterol rose to almost double the normal value while the esterified returned to normal. By the third day both free and esterified cholesterol were back to normal values.

These changes in rats induced by experimental interference with liver function agree well with the changes in plasma cholesterol in humans suffering from various liver disorders. Thus, in atresia of the bile duct and in acute yellow atrophy of the liver, there is an accumulation of free cholesterol in the plasma and a reduction of the esters (27). A drop in the ratio, [ester] / [total cholesterol], in the plasma appears to be a prominent feature of liver injury. Various interpretations have been suggested—deranged esterification in the liver, storage of esters in the liver (27), or faulty absorption from the intestine. It has been suggested that a significant alimentary hypercholesterolemia occurs only when the liver is injured (28). The removal of intravenously injected fat is delayed by liver injury (29).

In normal adult humans (30), the ratio of esterified to free cholesterol seems to vary only between the narrow range of 2.3 to 3.1 despite a range in total cholesterol of from 130 to 350 mg. per cent. In new-born infants, the total cholesterol values are generally lower than in adults, and the balance between esterified and free cholesterol is much more variable (31). Why the esterified cholesterol should remain constant at about 73 per cent of the total is rather perplexing, especially in view of the fact that there is an enzyme present in plasma which will esterify practically all of the free cholesterol if plasma or serum is allowed to incubate at 37 to 40° for

several days (32). Possibly it is of significance that the esterification is inhibited somewhat in the presence of tissue extracts (33). Esterification of cholesterol occurs on incubating colloidal solutions of cholesterol and free fatty acids to which a pancreatic powder and bile salts have been added. The bile salt protects the esterifying enzyme (34).

Changes in the ratio, [ester] / [free cholesterol] would seem to be of considerably greater clinical significance than changes in the level of the total cholesterol, which, even in normals, shows such a wide range of variation. In addition to the decreases observed in liver involvement, especially in inflammatory conditions, the cholesterol esters are found to be low in acute respiratory infections and pneumonia (35), and in depancreatized dogs (36).

It is a curious and, at present, rather baffling fact that such simple substances as methyl laurate and methyl undecylate, when injected intraperitoneally into rabbits, can induce a fall in the phospholipid of the blood with no change in the cholesterol, while methyl oleate decreases the blood cholesterol and increases the cholesterol excretion in the feces, the blood phospholipid remaining unchanged. Injection of methyl oleate into mice decreased the total cholesterol of the entire animal by 17 per cent (37). The physiological effects of these simple fatty acid esters is thought to have been responsible for the effects of certain extracts of the adrenal cortex.

DEPOSITION OF LIPIDS

In the fat depots.—It is well known that, under natural conditions, the depot fat of an animal is characteristic for the species. Certain inherent factors must exist which govern the nature of the depot fat. What these factors are, and especially how they operate, are still quite vague but the following would seem to be involved:

- a) The position in the scale of evolution (38).
- b) Environmental conditions. These would include temperature, which has been recognized for some years, and possibly salinity or at least marine conditions. Thus it has been found (39) that whereas fresh water and marine green algae and diatoms contain fats of essentially similar composition, the marine Crustaceae, which live upon the diatoms and algae, contain certain C_{20-22} acids, of a high degree of unsaturation, such as are not found in the food. The fresh water Crustaceae, on the other hand, apparently contain practically unmodified food fat.

c) Diet. It is a time-honored fact that the feeding of distinctive fats results in the deposition of large amounts of these food fats in the animal's stores. However, the extent to which the various ingested fatty acids are deposited is influenced by other factors in addition to their concentration in the food fat (40).

d) Saturation and desaturation of food fat. Desaturation of food fatty acids has been discussed above (page 247). In the case of certain marine fish whose food contains large amounts of very highly unsaturated fatty acids, there is evidence that these food fatty acids are saturated to some extent before (or soon after) being stored (41). For instance, in the various fat depots of the tunny there is an inverse relationship between the amount of stearic acid and the mean degree of unsaturation of the C_{18} acids. Furthermore, some arachidic acid is to be found in the liver, presumably as a result of saturation of some of the highly unsaturated C_{20} acids in the food (42). It must be emphasized, however, that both selective combustion and deposition of certain of the fatty acids in a mixture may account for differences in the composition of stored fat and of that in the food.

In the liver.—Accumulation of large amounts of neutral fat or of cholesterol esters or of both in the liver can be induced in several different ways:

a) By feeding a diet rich in fat, low in protein, and very low in choline (43). For example, on a diet containing 5 parts of caseinogen, 45 parts of glucose, 5 parts of salt mixture, 1 part of cod-liver oil and 40 parts of beef fat, in two weeks rats develop fatty livers containing about 24 per cent of total fat, the normal content being 3 to 4 per cent. Choline seems to be the most potent factor in preventing this deposition of fat, since the addition to the above diet of as little as 2 to 3 mg. of choline per day, either as such or in the marmite used as a source of vitamin B, will reduce the fat content to about 10 per cent (44, 45). On the other hand, it seems to be clearly established that certain proteins possess a so-called "lipotropic" action. Increasing the caseinogen content of the above diet from 5 per cent to 30 per cent reduces the fat content of the liver to about 6 per cent. Gelatin has no lipotropic action, while edestin does (44). On the other hand, feeding of additional cystine to rats on the low caseinogen, high fat diet increased the liver fat from an average of 22 per cent in the controls to 32 per cent (46). The nature of the fat fed appears to have an important bearing on the occurrence of

fatty infiltration into the liver; the degree of infiltration is thought to be roughly proportional to the percentage of saturated C_{14-18} acids in the food fat (47).

The manner in which choline prevents the deposition of fat in the liver and removes such deposits if they have already occurred appears to be still unknown. In view of the known participation of the liver phospholipids in fat metabolism and since choline is the base in lecithin, it has appeared likely to many that choline acts by driving the reaction, neutral fat \rightleftharpoons lecithin, towards the right. The fact that only small and frequently insignificant increases in liver phospholipids occur on feeding choline (48) is not a valid argument against such an interpretation since the newly synthesized lecithin may simply diffuse out into the blood stream as rapidly as it is formed. Attempts have been made to throw light on the mode of action of choline by feeding other related or homologous pentavalent nitrogenous compounds. Trimethylethyl-, trimethylphenyl-, and tetramethylammonium chloride increase the fat content of the liver instead of decreasing it (49). The triethyl homologue of choline, $\text{HOC}_2\text{H}_4 \cdot \text{N}(\text{C}_2\text{H}_5)_3 \cdot \text{OH}$, however, has a definite lipotropic action (50). The finding that the arsenic analogue of choline, $\text{HOC}_2\text{H}_4 \cdot \text{As}(\text{CH}_3)_3 \cdot \text{OH}$, appears in traces in the phospholipids of the brain and liver after being fed to rats (51) is very interesting and should be of great potential value.

b) Feeding cholesterol (approximately 2 per cent of the diet) together with enough fat to insure absorption causes an accumulation of cholesterol esters and neutral fat in the livers. It seems now to be established that the feeding of choline diminishes but does not entirely prevent the storage of cholesterol esters in the liver (48, 52). Whether this is a direct effect on the cholesterol esters or merely a consequence of the reduction in the neutral fat content of the liver is apparently a matter of opinion. Curiously, increasing the protein in the diet tends to accentuate the deposition of cholesterol esters in the liver (48). Because of the limited effect of choline on the deposition of cholesterol esters, the lecithin in dried liver (53) or dried egg yolk (54) does not suffice to prevent the storage of cholesterol esters on feeding large amounts of liver or egg yolk to rats.

c) In depancreatized dogs given insulin, after a period of some weeks or months, there is an enormous storage of fat and cholesterol esters in the liver together with a marked depletion of all the lipids and a practically complete disappearance of the cholesterol esters of the blood (36). The same is true of yellow atrophy of the liver (55).

The total lipids may account for over 60 per cent of the wet weight of the liver (56). The feeding of choline to such dogs reduces the accumulation of lipids in the liver (57) but does not restore the cholesterol esters of the blood (58). On the other hand, the feeding of raw pancreas prevents the fall and may even elevate above normal the blood cholesterol esters (58). The inference is that there is some substance in the raw pancreas other than choline which controls the liver and blood lipids, especially the cholesterol esters. This substance has been named "lipocaic" (57).

d) Extracts of the pituitary gland appear to induce storage of fat in the liver. Both pitressin and some factor in the anterior lobe (59), apparently the ketogenic hormone (60, 61), increase the infiltration of fat into the liver of fasting rats. One might think that overaction of this anterior lobe factor is possibly responsible for the accumulation of fat in the livers of depancreatized dogs. However, that does not seem to be the case (62) since hypophysectomy does not prevent the development of fatty livers.

Atherosclerosis, xanthomatosis, etc.—It is now well-established that feeding cholesterol, dissolved in oil, to rabbits and guinea pigs (but not to other laboratory animals) will produce a deposition of cholesterol esters in the aorta, thus simulating the atherosclerosis of humans. Recent studies (63) suggest that primary injury to the walls of the blood vessels is the chief cause of the deposition of lipids rather than the hyperlipemia and hypercholesterolemia. Attempts to induce such injuries and thereby to shorten the time required to produce atherosclerosis in the rabbit were unsuccessful (64, 65). On the other hand, the feeding of garlic oil reduced the hypercholesterolemia and the extent of cholesterol deposition in the aorta of rabbits fed cholesterol and oil (66). Potassium thiocyanate has also been reported to reduce the severity and incidence of atherosclerosis in thyroidectomized rabbits fed cholesterol (67). The lack of any correlation between the blood-cholesterol values and degree of atherosclerosis in human accident cases strongly indicates that the level of the blood cholesterol is not the primary cause of the disease, although it appears likely that it is a contributing factor (68, 69). Likewise there is no apparent relationship between the concentration of the various blood lipids and the occurrence of essential hypertension in humans (70, 71).

Recent evidence (72) has emphasized the fact that, whereas certain cases of essential xanthomatosis are improved by a diet from

which cholesterol-containing foods are excluded, others are not benefited.

Certain rather rare diseases are characterized by the accumulation of cerebroside or sphingomyelins in various organs of the body. Cerebroside cysts can also be formed (73). The study of the cerebroside content of the tissues of such cases may reveal significant facts (74).

The observation that the phospholipid which accumulates in the liver and spleen in Niemann-Pick's disease is sphingomyelin and not lecithin and cephalin (75), as was formerly believed, has been confirmed by others (76). A histological picture resembling that of Niemann-Pick's disease has been produced by injecting sphingomyelin into the blood stream of rabbits (77).

THE OXIDATION OF FATTY ACIDS

As a result of recent investigations, it has become necessary to consider seriously the possibility that there are four distinct mechanisms or pathways for the oxidation of fatty acids within the animal body:

(1) *Desaturation*.—This was proposed by Leathes many years ago. As mentioned above, the reality of the desaturation of saturated fatty acids within the animal body seems to be established (13). However, it is by no means proven that the desaturation of stearic acid to oleic (which appears most likely to have occurred) is primarily for the purpose of oxidation. The suggestion has frequently been made that the purpose of desaturation is to make possible the deposition of a fat which will be liquid at body temperature. The origin and function of the unsaturated C_{20-22} acids which are found so abundantly in the tissue phospholipids (78) and have been found to make up about 40 per cent of the total fatty acids in beef blood (79) are still quite obscure (see page 258 *et seq.*).

(2) β -*Oxidation*.—The essential correctness of this theory, as opposed to α -oxidation, has been further demonstrated by the finding that the odd-chain fatty acids, propionic, valeric, heptonic, and nonylic, all form glycogen in the liver of the fasting rat, whereas none of the even-chain fatty acids, with the questionable exception of butyric acid, does so (80).

(3) ω -*Oxidation*.—There can be no doubt of the fact that oxidation of the terminal methyl group of the saturated fatty acids with 8, 9, 10, and 11 carbon atoms in the chain does occur, with the result-

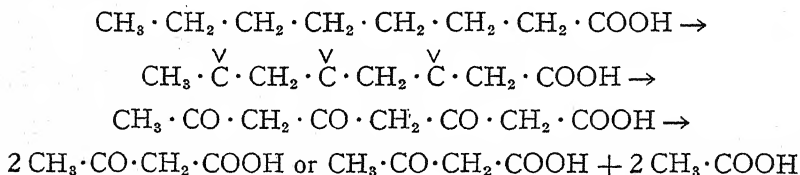
ant excretion of the corresponding dicarboxylic acids in the urine. [For a complete review see (81).] It has been shown also that β -oxidation of the resultant dicarboxylic acids occurs, since both adipic (C_6) and suberic (C_8) acids were isolated from the urine of dogs fed sebacic acid (C_{10}), while pimelic (C_7) and azelaic (C_9) acids were found after undecandioic acid (C_{11}) had been fed (82). Likewise monoethyl suberate was isolated after feeding monoethyl sebacate to a dog (83).

The outstanding question is to what extent ω -oxidation is to be considered as a major pathway through which the oxidation of all fatty acids proceeds in the animal body. Some (81) believe that all fatty acids, at least all the saturated fatty acids, may indeed undergo primary ω -oxidation. The reason that dicarboxylic acids are excreted only when C_{8-11} saturated fatty acids are fed is thought to be due to the greater resistance to oxidation of the C_{8-11} dicarboxylic acids than those with longer chains. This interpretation would seem to be borne out by the finding that, when given in equivalent doses to dogs, 45 per cent of suberic, 33 per cent of sebacic, 17 per cent of undecandioic, scarcely detectable amounts of brassylic, and no hexadecandioic acid were excreted in the urine. By ω -oxidation of the saturated fatty acids with subsequent two-sided β -oxidation, succinic acid will result. Since succinic acid can be converted to carbohydrate, it is suggested that this may be the long-disputed key to the question of the conversion of fat to carbohydrate (81). On the other hand, ω -oxidation as a primary pathway leaves no explanation for the formation of ketone bodies. Some (83, 84, 85) regard ω -oxidation as a special or auxiliary mechanism for the combustion of fatty acids which comes into play only when the carboxyl group is esterified or the β -carbon is blocked. The free carboxyl group thus formed makes possible a progressive β -oxidation. Under these circumstances, ω -oxidation certainly does occur. It has recently been shown that β -methyl caprate, when fed to rabbits, results in the excretion of β -methyl sebacic acid in the urine (86). The solution of the question must await further work.

(4) *Multiple alternate oxidation.*—By progressive β -oxidation of the even-chained saturated fatty acids, only one molecule of acetoacetic acid can be formed from each molecule of fatty acid, regardless of its length. Recent investigations have, however, brought forth evidence that more diacetic acid is formed from caprylic, capric, and lauric acids than from butyric or caproic acids. This holds for oxidation in the intact animal (87, 88) and in liver slices (89). For ex-

ample, when the ethyl esters of all the normal saturated fatty acids with even numbers of carbon atoms, from butyric to myristic, were fed daily to otherwise fasting rats, in doses equivalent to 15 gm. of acetone per sq. m. of body surface—calculated on the basis of 1 mol of acetone per mol of fatty acid—the average daily excretion of ketone bodies in grams per sq. m. was: from butyric 1.37, from caproic 1.95, from caprylic 7.29, from capric 6.74, from lauric 6.75, and from myristic 5.21 (88). When ethylacetoacetate was fed in the same equivalent dose, an average of 2.01 gm. (i.e., 13.4 per cent) were excreted. When injected intravenously into dogs, between 3 and 25 per cent of sodium acetoacetate was excreted, depending upon the concentration and the rate of injection (90).

To explain the greater production of ketone bodies from acids of eight or more carbons than from hexanoic and butyric, it has been suggested (88, 89) that there is a simultaneous oxidation of every alternate carbon atom with subsequent hydration and splitting, not only between the α - and β -carbons to form acetic acid as called for by the theory of β -oxidation, but also between the γ - and δ -carbons to form two molecules of acetoacetic acid, as illustrated by the following scheme (89):



The excretion of ketones after feeding the higher fatty acids (myristic, palmitic, and stearic) was greater than after the lower ones (88), a fact which was interpreted as evidence of the formation of three or four molecules of acetoacetic acid from each molecule of these higher fatty acids. The high ketone-body production with these normal food fatty acids has been claimed to be evidence against ω -oxidation as being a prominent pathway in fatty acid oxidation (88).

It will be noticed that the excretion of total ketones after an equivalent dose of caprylic acid (7.29 gm. of acetone per sq. m.) is more than twice as great as that after hexanoic (1.95 gm.) or after butyric (1.37 gm.) or after acetoacetic acid itself (2.01 gm.). Now, according to the above hypothesis, only two molecules of di-

acetic acid could be formed from caprylic acid. It follows, therefore, that a greater proportion of the *potential* acetoacetic acid is excreted when caprylic acid is fed (24 per cent if two mols are formed and 48 per cent if only one) than when hexanoic acid is fed (13 per cent). One wonders, in view of this greater excretion of the ketones formed from the long-chained fatty acids, if it may not be the explanation for the experimental findings.

It is noteworthy that the oral administration of ethyl myristate, ethyl palmitate, and ethyl stearate leads to the excretion of very considerable amounts of ketone bodies, whereas neutral fat does not do so. Fat absorption studies have led us to believe that the ethyl esters are hydrolyzed and the fatty acids linked with endogenously formed glycerol during absorption. It is not unlikely that the synthesis of glycerol from available carbohydrate accentuates the ketogenic action of the ethyl esters of the fatty acids.

The use of ketone-body production as an index of intensity of fatty acid catabolism would seem to be complicated by the fact that it is influenced by numerous other factors. Indeed, increased fat metabolism appears to have a very inconsistent effect on ketosis (91). It is well known that there is a ketogenic factor in the anterior pituitary which induces ketonemia and ketonuria. Its action is opposed by insulin (92, 93). The adrenal is also involved since an intense ketonuria develops after pancreatectomy but is abolished by adrenalectomy or hypophysectomy (94). Adrenalectomy abolishes the ketonuria of fasting pregnant rats (95), whereas the adrenal cortical hormone induces ketonuria in normal fasting females and males (96). There seems also to be good reason for believing that the presence or absence of ketonemia and ketonuria is dependent primarily on the condition of the liver and especially on the level of its glycogen content (93, 97, 98, 99).

Just how these various factors act and what relationship they bear to fat metabolism is not clear at present. Obviously the interpretation depends upon whether antiketogenesis is regarded as being due to the ketolytic action of simultaneous carbohydrate metabolism or to the catalysis of other catabolic reactions, thus taking some of the load off fatty acid metabolism (100).

THE PHYSIOLOGY OF THE PHOSPHOLIPIDS

Before proceeding with a discussion of the recent contributions to what is known about the phospholipids, it is perhaps advisable to

point out that the function of these tissue constituents is still unknown. It is quite clear (page 247) that part of the phospholipids in the blood plasma serves as a means of transporting fatty acids from the blood into the tissues where they are burned. This circulating metabolic phospholipid, so far as is known at present, does not accumulate to any extent in the various organs of the body (with the exception of the liver and, possibly, the intestinal mucosa) and therefore does not make up any appreciable part of the phospholipids present in such organs as the brain, the muscles, the kidneys, and the red blood cells.

It is not yet known where the circulating phospholipids are formed. It is customary to think of the liver. The finding of a rapid phospholipid metabolism and considerable changes in the amount (101) of phospholipids in the liver serves to substantiate such a view. The fact that the amount of phospholipid in the liver sometimes appears to remain quite constant (page 259) does not necessarily contradict the concept of a continuous turnover and, under certain conditions, storage of phospholipid. However, the finding that a high fat diet, involving a vigorous phospholipid metabolism, causes a fall in the phospholipid content of the liver, even when calculated on the basis of fat-free tissue, is difficult to explain (102). Increases under somewhat comparable conditions have been observed. On the other hand, it is known that there is a still more rapid turnover of phospholipid in the intestinal mucosa than in the liver and, although this has been interpreted as evidence that the phospholipids serve as an intermediary stage in fat resynthesis (page 246), it may be that the phospholipids of both blood plasma and liver originate in the intestinal mucosa.

The fact that the phospholipids of the hen's egg are a source of energy and of phosphorus for bone formation has recently been confirmed (103). The additional information has been added that the ratio of lecithin to cephalin in the yolk remains at 3:1 even though 62 per cent of the total phospholipid disappears during incubation. Of this amount, 19 per cent appears in the embryo. Whether there is a direct transfer is not known.

As for the phospholipids of the brain, the muscles, etc., any opinion at present as to their function is largely conjectural. Their physical properties suggest that they may constitute either the main or an important component of the cell membranes and of intracellular interfaces. Studies of the permeability of cells to various substances

lend considerable support to such a view (104). On the other hand, the phospholipids present in animal tissues always contain highly unsaturated fatty acids and many have had the idea that the double bonds serve as a reversible oxidation-reduction system and thereby function as agents for the transfer of oxygen within the tissue cells. It is not difficult to show that the unsaturated phospholipids are easily oxidized under approximately physiological conditions. Recently (105) egg lecithin was found to consume in six hours up to 76 per cent of its theoretical oxygen uptake in the presence of reduced glutathione and at pH 3.5. The fatty acids obtained after saponification had a much less rapid oxygen uptake. It is more difficult to demonstrate a reversible oxygen uptake by lecithin or to show that it has a catalytic effect in oxidation-reduction reactions. Recently it has been shown that the anaërobic reduction of *o*-cresolindophenol at pH 8.4 in the presence of oxidized glutathione is accelerated by the addition of lecithin and cephalin (105). However, the reduction of the dye under these conditions was also accelerated by the addition of hydrolecithin and of linoleic acid, so that the physiological implication is not as great as it otherwise would be. The antioxidant action of commercial lecithin, which has been used so extensively in certain food processes, has been found to be due to the cephalin content (106).

One of the fundamental concepts concerning the phospholipids has been that the amount of phospholipid in any one tissue is a fixed characteristic of that tissue, much as its histological structure is fixed. Many recent studies serve to strengthen this idea of constancy of phospholipid content. Thus, it has been found that the extreme hypertrophy of the heart and kidneys caused by feeding high protein diets to rats is not accompanied by any change in phospholipid (lipid P) content (107). Similarly the restricted growth of rats as a result of an inadequacy of either vitamin A or of calories does not lead to any changes in the phospholipid content of the various organs (108). Curiously enough, even a marked degree of fatty degeneration (as revealed by histological examination) of the livers of rats as a result of chloroform anesthesia is not accompanied by any significant change in the phospholipid content (109). All these findings suggest a rigorous constancy of the phospholipid content of tissues.

There is, however, much to be said in favor of the hypothesis that the phospholipid content of tissues is directly related to the physiological activity of the individual tissues and, therefore, is not a constant value. Most of the evidence in support of that hypothesis may

be found discussed in previous volumes of this *Review*. The recently found increase of 40 per cent in the phospholipid content of mice which were fed thyroid may be related to the increase in the oxygen consumption (110) in accordance with the above hypothesis, although any direct relationship between the oxygen consumption of a tissue and its phospholipid content has not been claimed to hold. However, there is a strong suggestion of such a relationship in the observation that the oxygen consumption and the phospholipid content of the skin of rats decrease in a roughly parallel fashion during growth. The apparent direct relationship found in normal animals does not hold for those suffering from a vitamin-B deficiency in which the oxygen consumption of the skin is greatly reduced while the phospholipid content is somewhat increased, as compared with rats of the same age (111). The increase in the phospholipid content of the placenta (112) and absence of any change in the ovaries (113) of the guinea pig have been interpreted as evidence in support of the concept of the relationship of phospholipid content to activity.

Since the phospholipid content of the individual skeletal muscles of various animals has been found to be a function of the amount of work which each muscle is customarily called upon to do (114), it seems reasonable to suppose that forced hyperactivity or hypoactivity of skeletal muscles would induce opposite changes in the phospholipid content from the normal values. However, analysis of the wing muscles of pigeons which either had been permitted to fly or had been confined to cages for several months did not show a sufficient difference to be regarded as significant, though the difference was usually in the expected direction (114). The fatty acids of the phospholipids from these various muscles were fractionated into solid and liquid acids and their iodine numbers determined. There was no indication of a difference in the composition of the phospholipids other than could be attributed to differences in diet, except in those from the forearm of the exercised pigeons which contained more highly unsaturated fatty acids (I.No. 147) than those of the confined pigeons (I.No. 128). Whether this is an adaptation to take care of an increased strain on the functional capacity of the phospholipids must be left for future work to settle.

Recent studies (115) have confirmed the earlier findings that cephalins are highly active in accelerating blood clotting, whereas purified lecithins are not. The preparation, analysis, and testing of the phospholipids of the blood platelets has been accomplished for the

first time. Their total phospholipid content amounts to 12 per cent of the dry weight. On the basis of moist weight, blood lymphocytes contain about 0.6 per cent and the neutrophils about 2 per cent of phospholipid (116). Additional evidence has recently been published which indicates that the phospholipids may play an important rôle in immunological reactions. Furthermore, lecithin appears to be the phospholipid component of the specific precipitate from antipneumococcus horse serum, and cephalin of that from rabbit serum (117).

THE PHYSIOLOGY OF CHOLESTEROL

Most of the recent findings concerning the variations of total cholesterol in the body and the balance between the free and esterified forms have been discussed. A few observations of considerable significance remain.

As to the function of cholesterol in the body, there is still almost complete ignorance. No one has as yet been able to formulate a thoroughly satisfying hypothesis as to why all tissues contain free cholesterol; why the amount varies from one tissue to another and yet appears to maintain a relatively constant value in each individual tissue; why the amounts of phospholipid and free cholesterol appear to maintain a relatively fixed ratio to one another, even though the value of that ratio differs markedly from one tissue or organ to another; and what metabolic relationship exists between cholesterol and the other substances with the same fundamental skeleton—the bile acids, the sex hormones, etc.

In the hope that an answer might be found for some of the above questions, a systematic study has been made of the cholesterol and the phospholipid content of a large number of muscles, both smooth and cross-striated, from an extensive series of cold- and warm-blooded animals (118). It has been found that the three conventional types of muscles show definite differences in cholesterol content and [phospholipid]/[cholesterol] (P/C) ratio. Smooth muscle has the highest cholesterol content (average 0.8 per cent) and the lowest P/C ratio, viz. 4; cardiac muscle in most warm-blooded animals has the next highest cholesterol content (0.5 per cent) and a relatively high P/C ratio, viz. 14; but in some animals (man and all the cold-blooded animals) the cholesterol content of cardiac muscle is as high or even higher than that of smooth muscle, with a drop in the P/C ratio; the skeletal muscles (thigh) as a rule have the lowest cholesterol con-

tent (0.3 per cent) and the highest P/C ratio, viz. 16. In the same animal different skeletal muscles have about the same cholesterol content. While there is a considerable range of variation in the values found for the muscles of each group, with some overlapping, nevertheless there would appear to be a relationship between the normal functional activity of a muscle and its cholesterol content and P/C ratio. The automaticity of smooth and cardiac muscle is thought to be related to the high cholesterol content (118).

It is now generally believed that there is an active cholesterol metabolism at all times within the animal body, the total amount present being the expression of the dynamic equilibrium existing between cholesterol absorption and synthesis on the one hand, and excretion and destruction on the other. The mechanisms and pathways of the synthetic and catabolic processes are still quite obscure. As for excretion, there is good evidence that it involves continuous secretion of cholesterol in the intestinal tract, part being reabsorbed and part excreted with the feces. Normally, there is an extensive conversion of cholesterol into coprosterol within the intestine as a result of the activity of the bacterial flora. In the dog (119), a low meat diet decreases and a high meat diet increases the formation of coprosterol; the same does not seem to be true of rats, in which the ratio of the saturated to unsaturated digitonin-precipitable sterols is the same, regardless of the diet (120).

Since hydrogenation of cholesterol *in vitro* results in the formation of dihydrocholesterol rather than in the isomer coprosterol, there has been much speculation as to the mechanism whereby the intestinal bacteria convert cholesterol into coprosterol. Two possibilities have been suggested: conversion of cholesterol into allocholesterol and then reduction to coprosterol; oxidation of cholesterol to cholestenone, followed by reduction first to coprostanone and then to coprosterol. Since allocholesterol cannot be found in the body, a fact recently confirmed by a new method of approach (121), the second possibility has appeared most likely. Further evidence of the intermediary formation of cholestenone from cholesterol has been obtained by feeding cholestenone to dogs on a low and a high meat diet and recovering, from the feces, cholesterol and coprosterol, respectively. Furthermore, coprostanone was formed from cholestenone by saturating the double bond with deuterium; on feeding this coprostanone to a human subject and to dogs, coprosterol containing deuterium was recovered from the feces (119).

THE FORMATION OF MILK FAT

For many years blood phospholipid has been thought to be the probable precursor of milk fat, the phosphoric acid-base complex being replaced by a fatty acid molecule through the activity of the mammary gland. More recent investigations, involving the use of an improved technique, have seriously questioned the correctness of this belief. Within the past year, the origin of the fat and some other constituents of cow's milk has been reinvestigated, use being made of a new technique for obtaining samples of blood from the internal iliac artery (122). The concentrations of the total fatty acids, lipid phosphorus, and cholesterol were determined in samples of blood drawn from the artery and from the mammary vein. There was no significant difference in the concentrations of lipid phosphorus and cholesterol in the two samples of plasma or of whole blood. The total fatty acids, however, in most of the experiments were lower in mammary venous blood than in arterial blood. The average difference amounted to 4.1 mg. per cent in one series of twenty experiments and 7.2 mg. per cent in a second series of nineteen experiments. Expressed as percentages of the arterial blood values, these differences amount to 1.9 per cent and 3.5 per cent, respectively, which, while rather small, seem to be significantly greater than the probable error of the method as used by the authors. Furthermore, since there was no detectable withdrawal of phospholipid from the blood, it would appear to be almost essential to believe that the fat or cholesterol esters must be the source of the milk fat. The withdrawal of sugar was scarcely, if any, greater than that necessary to provide the sugar for the milk.

In an accompanying paper it is demonstrated that the injection of thyroxine into lactating cows increases both the volume and the fat content of the milk, the daily fat output being increased by 50 per cent (123).

Milk fat is unique among the animal fats in having the complete range of C_{2n} fatty acids from butyric (C_4) to cerotic (C_{26}) (124, 125). In fact those fatty acids with fourteen or less carbon atoms in the molecule make up about 20 per cent of the total (126). The origin of these short-chain fatty acids is an interesting problem. Apparently they do not occur in the blood in detectable amounts (79). It appears likely that they are formed in the cells of the mammary gland. The tentative suggestion has been made (126, 127) that the shorter-chain fatty acids are formed by oxidative degradation of the longer

fatty acids, beginning at the terminal $-\text{CH}_3$ group, with subsequent reduction of the terminal carbon atom. The basis for this suggestion is the finding in butter fat of decenoic and tetradecenoic acids in which the double bond is in the same 9:10 position as in oleic and linoleic acids. It is implied too that the oxidation and reduction of the fatty acids occur while in combination with glycerol, thus offering an explanation for the high content of fully saturated glycerides in butter fat.

Since the fatty acids from the blood are probably used by the mammary gland cells as sources of energy as well as for the production of their specific secretion, one wonders if the milk fatty acids may not give some insight into the manner in which fatty acids are oxidatively degraded in other tissue cells.

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THE METABOLISM OF PROTEINS AND AMINO ACIDS*

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AMINO ACIDS

Oxidation of the amino acids.—The problem of oxidation of the amino acids has been studied by Bernheim, Bernheim & Gillaspie (1) in an important work. The oxidation enzyme was isolated in purified form from kidneys. Contrary to the experiments with organ slices the enzyme in solution was only able to oxidize the foreign isomers of the amino acids. The formed ketonic acids were isolated as phenylhydrazones or bisulfite compounds and were estimated by titration with iodine. In all cases the methods gave a yield of more than 50 per cent. During the oxidation of the foreign isomers it was noticed that methemoglobin was formed. This was shown to be due to the formation of hydrogen peroxide which caused the oxidation of hemoglobin to methemoglobin in the presence of the enzyme preparation. *dl*-Alanine, *l*-valine, *dl*-serine, *d*-leucine, *l*-isoleucine, *d*-phenylalanine, *dl*-methionine, *dl*-proline, *l*-proline, and *d*-histidine were examined. The oxidation product of *d*-tyrosine gave no phenylhydrazone, and the enzyme preparation oxidized neither the natural nor the foreign isomer of aspartic acid; proline behaved exceptionally.

The oxidative deamination of amino acids has also been studied by Neber (2). Starting from the well-known investigations of Krebs (3) he was able to prove that, first of all, the liver, then the kidneys, and next the intestinal mucous membrane must be considered as the most important places of decomposition. This confirms the findings of London (4). With the exception of glycine the simpler amino acids are the most easily catabolized. Contrary to nutrition experiments the foreign components are found to be broken down more rapidly than the natural isomers by surviving slices of all three organs. Tryptophane and *d*(+)-histidine are scarcely attacked by the kidney.

Hydroxyamino acids.—Meyer & Rose (5) have found that α -amino- β -hydroxy-*n*-butyric acid is a cleavage product of proteins and have demonstrated that the new amino acid is an indispensable component of the diet. They made a study of its spatial configuration. The results demonstrate that this constituent of proteins is a

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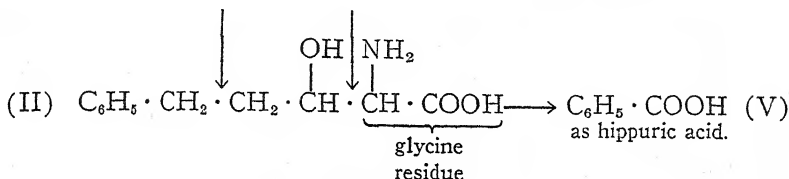
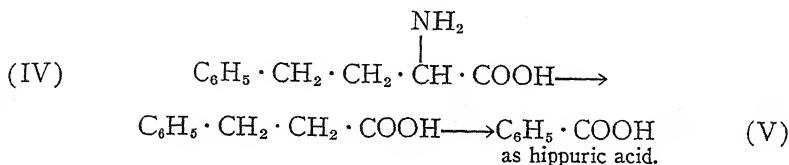
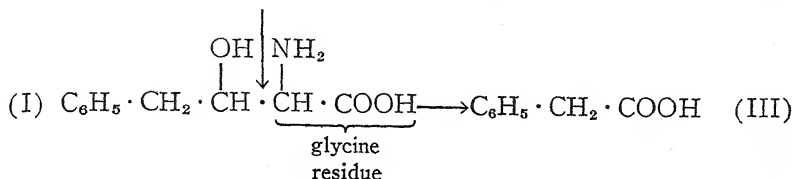
compound analogous in structure to *d*(—)-threose. Because of this relationship the amino acid has been named *d*(—)-threonine.¹

Knoop *et al.* (6) have inquired into the occurrence and metabolism of hydroxyamino acids in the animal body. The importance of this group of amino acids had already been realized by the discovery of β -hydroxy- α -aminobutyric acid by Rose *et al.* (7). As hydroxyamino acids are very sensitive they may decompose during protein hydrolysis and their isolation be rendered impossible. On heating with mineral acids hydroxyglutamic acid, e.g., is converted into α -ketoglutaric acid. It is, therefore, possible that hydroxyamino acids, in greater quantities and of different configuration than has been accepted hitherto, must be considered as constituents of the proteins. In consequence an inquiry has been made as to whether β -hydroxy- α -amino acids are decomposed like the simple amino acids or whether their metabolism proceeds differently.

γ -Phenyl- β -hydroxy- α -aminobutyric acid (I), as well as δ -phenyl- β -hydroxy- α -aminovaleric acid (II), were prepared by synthesis and administered to a dog. Following the administration of the phenylhydroxyaminobutyric acid, phenylacetic acid (III) appeared in the urine, whereas the simple γ -phenyl- α -aminobutyric acid (IV) was converted into benzoic acid (V) in the well-known manner. In an analogous way phenylhydroxyaminovaleric acid yielded benzoic acid exclusively; this was isolated in the form of hippuric acid.

The decomposition of the β -hydroxy- α -amino acids therefore does not take place following the laws of metabolism of the simple amino acids in which fatty acid formation is followed by β -oxidation; here, β -oxidation takes place directly. This fact definitely shows that the β -hydroxy- α -amino acids can also be abnormal intermediate products of amino acid metabolism. It may also be conceived that the hydroxyamino acids are potential sources for formation of glycine. On decomposition of the hydroxyamino acids the corresponding β -keto- α -amino acids must be considered as intermediate products. These substances show a higher redox potential than ascorbic acid and they reduce many dyes to leuco bases. Therefore, they play an important rôle in oxidative processes in metabolism.

¹ In former nutrition experiments with mixtures of amino acids, Womak & Rose (5) showed that phenylalanine is indispensable for life and incapable of being replaced by tyrosine. Analogous experiments with mixtures of amino acids, also containing threonine, show that leucine and isoleucine are indispensable whereas norleucine does not seem to be essential for growth.



In the paper cited (6) the synthesis of both hydroxyamino acids is described and it is shown that serine and isoserine can be differentiated by means of the Criegee reaction with lead tetra-acetate. Martius & Knoop (8) determined the redox potential of the above mentioned α -amino- β -ketobutyric acid; with an E_0 -value of 0.077 mv. the ester of the keto-aminobutyric acid is one of the strongest reducing substances.

Malherbe (9) found that the only amino acid oxidized by brain is $l(+)$ -glutamic acid which is oxidized to α -ketoglutaric acid and ammonia and further to water and carbon dioxide. The enzyme responsible for the oxidation of the $l(+)$ -acid does not attack $d(-)$ -glutamic acid so long as it is bound in the cells, probably to a lipid or to some constituent of the cells. In solution, however, the specificity is changed and $d(-)$ -glutamic acid alone is oxidized. The oxidation of $l(+)$ -glutamic acid to α -ketoglutaric acid is reversible. The view is expressed that, *in vivo*, the glutamic acid deaminase is concerned, rather, with the synthesis of glutamic acid.

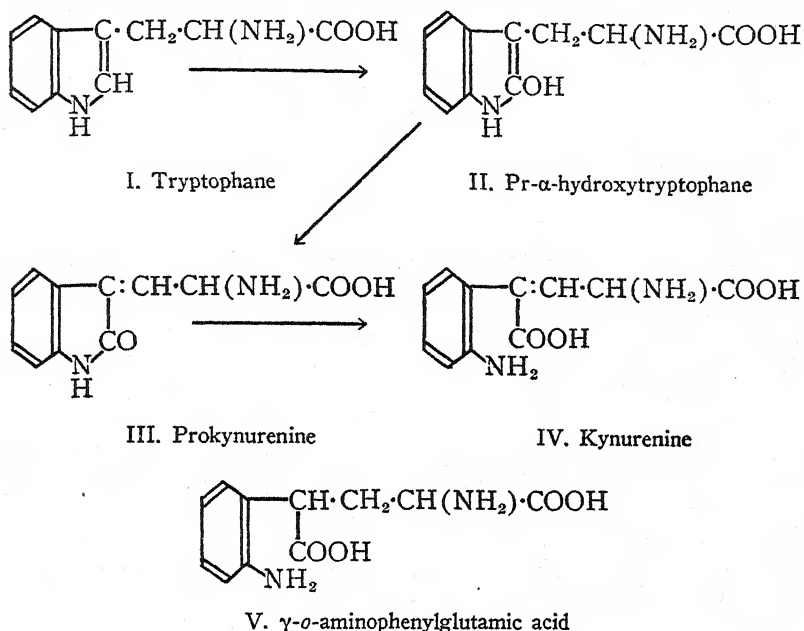
The rôle of amino acids in the metabolism of strict anaërobes (Clostridium).—Woods (10) has pursued investigations on this subject in continuance of the former work of Stickland (11). Through coupled reactions between pairs of amino acids Woods could show that only d -arginine and d -ornithine may act as hydrogen donators.

Both substances may be activated as hydrogen acceptors but in addition are partially deaminated in the absence of hydrogen donors. When ornithine reacts with the donor alanine, it accepts two hydrogen atoms and undergoes reductive deamination to δ -aminovaleric acid. Glycine, β -alanine, and taurine are not attacked by the organism.

The oxidation of amino acids by *B. pyocyaneus* is reported upon by Webster & Bernheim (12). Tryptophane and methionine are not attacked. Only the natural components of leucine, isoleucine, and histidine, and both components of alanine, serine, tyrosine, and proline are attacked; the natural isomers of valine and phenylalanine are oxidized and, to a lesser degree, the foreign isomers as well.

Tryptophane.—Kotake & Masayama (13) assume that the decomposition of tryptophane takes place along different pathways. One mode of catabolism begins at the side chain with deamination. According to a second mechanism tryptophane is attacked in the indole ring by opening of the pyrrole nucleus. Decomposition is also possible by the opening of the benzene ring. In former communications (14) the second mechanism, leading to the formation of kynurenine, has been described. Kotake & Ichihara (14) are of the opinion that kynurenic acid is not an intermediate product of tryptophane metabolism, but that the formation of this acid is to be considered as a detoxication process which becomes operative when there is an excess of tryptophane or kynurenine. It has been shown that kynurenine is formed by digestion of tryptophane with liver slices or with raw liver extract. Thereby, a prokynurenine seems to be formed; this is transformed into kynurenine. Tryptophane (I) is first of all converted into pr- α -hydroxytryptophane (II) which is now assumed to be transformed into prokynurenine (III) and finally into kynurenine (IV). The authors consider this mechanism to be more probable than the hitherto-assumed transformation of pr- α -hydroxytryptophane (II) via γ -*o*-aminophenylglutamic acid (V) into kynurenine (IV). Thus there exists a special enzyme in the liver of rats, rabbits, etc., which converts prokynurenine [III (also named kynureninlactum)] into kynurenine with the addition of water. It is possible that the formation of anthranilic acid from tryptophane by bacteria also takes place via kynurenine. The foreign *d*-tryptophane forms only prokynurenine and not kynurenine. That on decomposition of *l*-tryptophane two substances, prokynurenine and kynurenine, are formed can be shown by the fact that one of them, after making slightly alkaline with baryta, gives, on heating, amino-acetophenone with its charac-

teristic jasmine-like smell; while the other, only after heating in a concentrated potassium carbonate solution, shows the same reaction.



Ichihara & Nakata (15) show that in the nutrition of rats *dl*-indolelactic acid can replace tryptophane, whereas it was already known that *d*(—)-indolelactic acid is not able to do this. From the racemic indolelactic acid *l*(+)-indolelactic acid was prepared by means of the quinine salt and is now shown to be twice as active in nutrition as the racemic form. In accordance with Knoop's theory it is assumed that the hydroxy acid is transformed through the keto-acid into tryptophane and it follows, therefore, that only the *l*(+)-indolelactic acid in the organism may be converted into tryptophane.

Majima (16) examined the formation of indole from tryptophane by *Bact. coli*. He found that the formation from *l*-tryptophane is quantitative. Dextrorotatory indolelactic acid gives indole quantitatively, in contrast to the levorotatory form. It is supposed that the dextrorotatory indolelactic acid, on account of its relationship to the natural *l*-tryptophane, has to be formulated as *l*(+)-indolelactic acid.

According to Woods (17) racemic tryptophane is asymmetrically decomposed by *Bact. coli*. Majima (18), by means of this method, succeeded in preparing *d*-tryptophane.

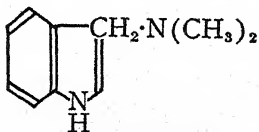
In continuing these experiments Kotake, Ichihara & Nakata (19) examined the different utilizations of *l*- and *d*-tryptophane in rats and mice. It was shown that *d*-tryptophane and *l*(+)-indolelactic acid in rats act just as well as *l*-tryptophane, in contrast to similar experiments with mice. The authors assume that the rat is able to transform *d*-tryptophane into the *l*-form. This physiological "*Umbildungsfähigkeit*" is also demonstrable on feeding with methionine.

Ichihara & Goto (20), confirming the work of Gordon *et al.* (119), examined different animal species in respect to their ability to excrete kynurenic acid in urine and bile. Whereas this acid could not be detected in the bile of man, cat, or fowl, it could be found in human urine.

Hamada (21) examined the hematopoietic action of tryptophane and observed that in young rats, after a tryptophane-free diet, a certain degree of anemia occurs which can be improved by administering small quantities of tryptophane; the effective doses are so small that they cannot be considered as real food. The question whether tryptophane serves as a formative substance or as a stimulant could not be decided. *d*-Tryptophane had no hematopoietic action.

The typical effects of tryptophane on growth and on formation of kynurenic acid have been examined by Bauguess & Berg (22). These authors administered carbomethoxy-*N*-tryptophane (-ethoxy-, propoxy-, -phenoxy-, benzoxy-). None of these substances was active.

The tryptophane content of leguminous plants in different stages of growth has been examined by Virtanen & Lane (23). They find that it increases considerably in periods of rapid growth, e.g., before blooming. This may be brought into causal connection with the formation of indole-acetic acid. Löfgren (24) determined the content of tryptophane in the germinal layers of barley in an effort to establish a connection between the formation of indole derivatives and gramine [isolated from the leaves of barley by Euler (117)]. A lower content of tryptophane was found to be associated with defective chlorophyll formation. Wieland & Hsing (116) report on a synthesis of gramine according to which the following formula may be assigned:



Histidine.—Földes (25), employing a modification of Kapeller-Adler's method (26), studied the histidine content of urine. He found

considerably more histidine in the urine of gravid than in that of non-pregnant women. The reaction, however, is not specific. Urine of non-pregnant animals does not contain histidine. In more recent studies Kapeller-Adler (27) has worked out an improved method of determination. Many urines contain nitrites which, first of all, have to be eliminated by oxidation with permanganate. According to this new method the error is only 2 to 3 per cent.

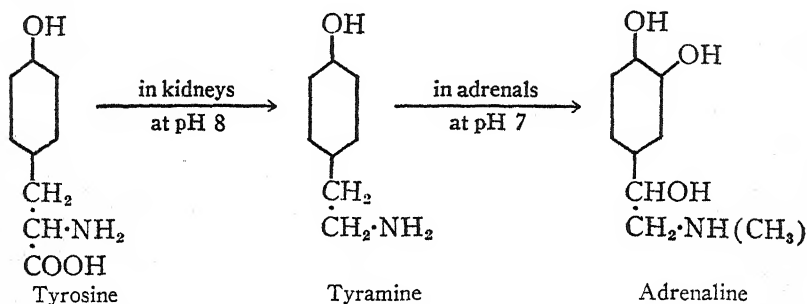
Formation of histamine from histidine.—Bloch & Pinösch (28) examined the organs of guinea pigs for histamine. As other authors had found, they demonstrated that the lungs are the chief reservoir for this substance. The histamine-decomposing enzyme, histaminase, is not present in the lungs. The effect of histidine on the histamine stored in the lungs was also studied. Guinea pigs were given 0.1 gm. of histidine per 100 gm. body weight subcutaneously. After five hours the lungs were examined for histamine by means of the guinea-pig intestine method. While the normal histamine content of the lungs was 15 to 25 $\mu\text{g.}^2$ per gm. of tissue, after the injection of histidine it was increased to from 40 to 45 $\mu\text{g.}$ per gm. Arginine, glutamic acid, aspartic acid, valine, glycine, alanine, sodium chloride, and even prolonged fasting, had no influence whatever. From these experiments it must be considered as highly probable that histidine in the animal body is transformed by decarboxylation into histamine which is then stored in the lungs. The metabolism of histidine is limited, on one hand by histidase, found by Edlbacher in the liver, and on the other hand by the formation of histamine. Werle (29) found that surviving slices of kidney apparently accomplish the decarboxylation of histidine into histamine. [See also: Anrep, Barsoum & Talaat (30) and Barsoum & Gaddum (31).]

Carnosine.—Du Vigneaud & Hunt (32) described a synthesis of the foreign *d*-carnosine and found that the injection of even twenty times as much of the *d*-compound as of the *l*-compound caused no depressor effect. This action is peculiar to the natural *l*-carnosine.

Tyrosine.—Schuler, Bernhardt & Reindel (33) investigated the fate of phenylalanine and tyrosine, and came to the conclusion that tyrosine must be the mother substance of adrenaline. Slices of surviving tissue were used. The conversion does not take place in tissue extracts. Surviving slices of guinea-pig kidney decarboxylate tyrosine into tyramine. This decarboxylation reaction is not achieved with liver tissue. The experiments must be undertaken in such a way that

$^2 1 \mu\text{g.} = 0.001 \text{ mg.} = 1 \gamma.$

the tissues are not too strongly oxygenated, otherwise an oxidative deamination takes place. The formation of adrenaline itself does not take place in the kidneys but in the adrenals which are unable to form tyramine and must be supplied with that substance.

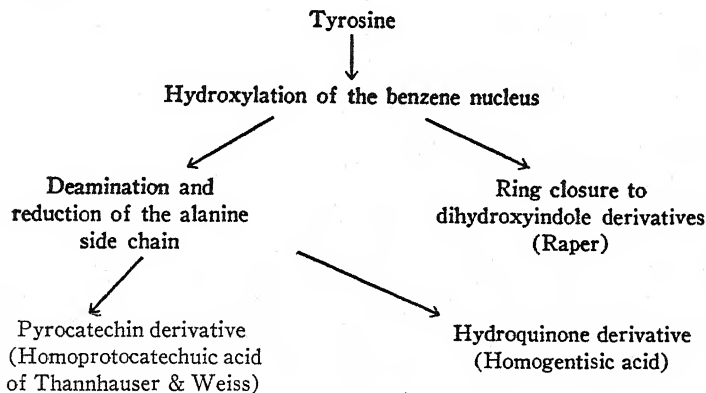


Phenylalanine and phenylethylamine were inactive. The adrenaline formed was identified as such by a colorimetric method and by its pressor activity in the narcotized animal. In parallel experiments it was shown that putrefactive bacteria do not accomplish the decarboxylation of tyrosine, for if liver slices were digested with tyrosine at 38° during two days the solution failed to decarboxylate tyrosine under the chosen conditions. Kidney pulp or powder does not attack tyrosine. Only surviving slices of kidney accomplish the decarboxylation and that, as already mentioned above, under access of air but not of oxygen; the optimum pH is 8.0. On the other hand Schuler & Wiedemann (34) showed that the formation of adrenaline from tyramine in the adrenals takes place at an optimum pH of 6.8 to 7.4. Thus the kidney is able to deaminate tyrosine oxidatively in the presence of an excess of oxygen, whereas under limited oxygen supply it accomplishes the decarboxylation. In former work Bernheim (35) showed that the liver decomposes *l*(-)-tyrosine oxidatively and does not decarboxylate this substance. Schuler *et al.* assume that this decomposition of tyrosine in the liver is necessary for destroying the excess of tyrosine which cannot be converted into adrenaline. This is analogous to the histidine decomposition in the liver by histidase as described by Edlbacher & Neber (36).

Holtzmann (37) has studied the tyrosine balance of *Bombyx mori* (silkworm) in respect to the manufacture of silk. The tyrosine intake in mulberry leaves ingested by silkworms was found to exceed the output of tyrosine in the silk and the contained pupae; hence there is no need to postulate a mechanism for the synthesis of tyrosine.

In this connection reference should be made to the work of Abelin (38) on the action of di-iodotyronine, di-iodotyrosine, and thyroxine. Cavett (39) investigated the composition of human thyroglobulin and found that in preparations from normal and goitrous glands the quantities of the different amino acids are the same except in the case of thyroxine, tyrosine, and di-iodotyrosine. In thyroglobulin from colloid goiter the content of iodine-containing amino acids seems to be decreased, whereas it increases after administration of iodine [compare with Alcock (40)].

Melanine.—Barrenscheen & Prinz (41), in continuing former work, studied the question of melanogens and melanine. They worked out a method by which ether-soluble and ether-insoluble melanogens can be separated. An ether-soluble melanogen, free of nitrogen, could be obtained in crystalline form; it was not a pyrocatechin derivative. In a detailed manner they describe the isolation of ether-insoluble melanogens from urine and tumors and come to the following conclusion on the formation of the nitrogen-containing ether-insoluble melanogens:



Zeynek & Waelsch (42) made similar studies of Thormählen's reaction in melanotic urine. Nobutani (43) examined the rôle of different phenols in the formation of pigment by potato tyrosinase. He found that amino acids activate the absorption of oxygen and assumes that the amino acids form compounds with the *o*-quinones.

Lysine.—Berg (44) prepared for the first time the foreign *l*(+)-lysine from synthetic *dl*-lysine by means of camphoric acid.

Arginine.—In 1928 Felix & Müller (see 45) prepared α -hydroxy-*d*-guanidinovaleric acid by the reaction between silver nitrate and

arginine, in connection with the preparation of iminazolelactic acid from histidine (46). They examined the behavior of this compound, called "argininic acid," in metabolism. It was administered to rabbits and dogs, and to men with pseudohypertrophic muscle dystrophy, myasthenia, and diabetes. In the animal and in the cases of muscle dystrophy it caused an increase of the urinary creatine which partially depended upon new formation. The creatinine excretion in myasthenia was not influenced. In the diabetic organism it was for the most part converted into urea and sugar. Following Brand, Harris, Sandberg & Ringer (47), patients with pseudohypertrophic muscle dystrophy are particularly indicated for this kind of experiment as they cannot retain creatine.

Edlbacher & Zeller (48) examined the decomposition of arginine by arginase. In former investigations on the action of liver extract only the natural *d*-component was split, whereas in the surviving liver, in perfusion experiments with racemic arginine, a complete decomposition of arginine took place [see also Felix & Morinaka (49)]. It has now been shown that a several-fold increase in enzyme concentration is responsible for the complete decomposition of *l*-arginine into urea and ornithine. It must be concluded from this that the optical specificity of the enzymes which split amino acids is a function of the enzyme concentration. By generalizing the results found with arginase a series of discordant results reported in the literature can be explained. The findings are also in agreement with Krebs' statement (50) regarding oxidative decomposition of foreign amino acids.

The activation of arginase by manganese salts in respect to optical specificity and species differences as described by Klein (51) has been examined. Hellermann & Perkins (118) reported similar activation experiments on arginase and various metallic salts, and found that manganese only promotes the cleavage of the natural *d*-arginine. It was further shown that different tissues of birds, which normally contain scarcely any arginase, were strongly activated by manganese. From these experiments it must be concluded that the avian organism is potentially capable of forming urea; the hypothesis that Schuler's *Harnsäure Vorstufe* possibly begins with the formation of urea (in accordance with Wiener's theory) is advanced. Finally it is shown that the different amino acids and urea strongly inhibit arginase.

Heinsen (52), on working up an autolysate of ox kidney (toluene and chloroform) after ten days' autolysis at 40° (pH 3.8), found a small quantity of racemic arginine.

Hirai (53) examined the formation of putrescine from arginine by bacteria and found that *Bact. coli* convert *D*-arginine into putrescine.

In respect to the blood arginase Anderson & Tompsett (54) come to the following conclusion:

The reported production of "extra" urea when human blood is incubated with concentrated solutions of urease is confirmed. The substance responsible is considered to be the blood arginase. It is confined to the corpuscles and is inactivated by incubation at 37–50° for 10 min. after the addition of acid to the blood and is activated by the addition of cobaltous ions which may produce an increase of 100% in the apparent blood urea. A similar production of ammonia is observed when the concentrated urease solutions are incubated with a preparation of liver arginase. The significance of this error in urea analysis is discussed and the use of plasma is advised for the accurate estimation of urea in human blood.

In a number of investigations Bayerle, Barger & Mayr (55) examined artificial renal infarcts and could state that in respect to arginase a fundamental difference exists between autolysis and necrosis.

Hexone bases.—Edlbacher & Baumann (56), in pursuing former work on the protein metabolism of tumors, studied the content of hexone bases in the Jensen sarcoma of the rat and in tumor necrosis. Drummond (57) had made similar experiments. Edlbacher & Baumann found that there is no difference between the hexone-base content of tumor tissues and the necrotic material. This is the more remarkable as the purine content of the necrotic tissue is only, at most, one-sixth of the purine content of the intact sarcoma tissue, as Edlbacher & Jucker (58) showed. In these experiments it is also pointed out that the purine content of the organs of the rat, after maintenance on an unbalanced diet, shows great fluctuations. After abundant over-nutrition, e.g., with proteins, the purine content of the liver and the kidneys is appreciably diminished.

Creatine.—Beard & Boggess (59), in continuing their former work, examined the formation of creatine from amino acids injected into the rat. Creatinine, arginine, histidine, glycine, alanine, serine, and valine were used. The creatine content of muscle, stomach and liver from one to four days after injection was determined. The increased creatine formation observed in these studies was probably not the result of a "stimulation" process, either of the endogenous metabolism or of creatine formation. When the amino acids cause an increase of muscle creatine up to 50 per cent above normal, and when it is

recalled that glycine and glutamic acid may cause increases up to 1000 per cent in urinary creatine in some myopathies, it is clearly seen that creatine metabolism in the organism is unstable and may be easily affected by many factors (proteins, amino acids, nuclear material, choline, various hormones, muscular diseases, fever, etc.). Therefore, it is believed that creatine arises in the body from its exogenous precursors, chiefly proteins and amino acids. Although the exogenous origin of creatine from amino acids is not accepted by many students of creatine metabolism at the present time, it should be remembered that most of the negative results have been obtained in experiments in which small doses of amino acids were fed or injected and the excretion of creatine and creatinine in the urine was used as an index of creatine formation in the body. In these cases creatine may have been formed and stored in the muscles and not excreted in the urine. Also, at the present time, some workers are beginning to deny the existence of any metabolic relationship between creatine and creatinine. Thus it hardly seems necessary to point out that creatine formation in the muscles is an entirely different physiological process from the production and excretion of urinary creatine and creatinine. It is well known, especially from clinical studies in myopathies, that ingestion of amino acids may increase the excretion of creatine and creatinine.

Creatine formation from its precursors occurs chiefly in the muscles and maximum increases are observed two days after injection. Goudsmit (60) also examined the origin of the urine creatine. By comparing the creatine contents of venous and arterial blood it was found that in the kidneys a substance occurs which is to be regarded as the chromogen of Jaffe's reaction. Bodansky (61), after feeding creatine, guanidino-acetic acid, and glycine to rats, studied the liver, kidneys, heart, and muscles. In the liver the creatine content increased 400 to 500 per cent after feeding with 50 to 100 mg. of creatine, and returned to normal after twelve to twenty-four hours. After feeding guanidino-acetic acid, up to 50 per cent of that absorbed was excreted in the urine; at the same time the creatine content of the kidneys increased. It seems, therefore, as if guanidino-acetic acid were a precursor of creatine.

Carnitine.—Stark, Wördehoff & Schwaneberg (62) studied the significance of carnitine in muscle. A simplified method of preparation from meat extract was worked out so that about 18 per cent of this material could be extracted. Pure carnitine and acetylcarnitine

have very little biological action on the rectus muscle of mice and frogs and very probably play no rôle as stimulants of muscle contraction (182 mg. have the same activity as 3 μ g. of acetylcholine). There exists, however, a substance mixed with carnitine which has, in acetylated form, a strong biological action. The action of this unknown substance can be inhibited by atropine and increased by physostigmine. It can give rise to errors during the biological estimation of carnitine in muscle. Weger (63), in the same way, examined the action of carnitine and acetylcarnitine. He found that solutions which contain 0.02 to 0.15 per cent of acetylcarnitine have a distinct inotropic action on the frog heart and that fresh extracts of frog heart abolish the activity of acetylcarnitine.

Creatinine.—At the suggestion of Terroine, Mourot (64) has studied creatinine excretion and has shown that this excretion is related to the degree of protein conversion. Terroine, Bonnet & Mourot (65) examined then the creatinuria under different nutritive conditions.

Guanidine.—Ackermann (66) examined the fate of taurocyamine [guanidyltaurine, $\text{HN}:\text{C}(\text{NH}\cdot\text{CH}_2\cdot\text{CH}_2\text{SO}_3\text{H})\cdot\text{NH}_2$] in the dog. It was supposed that it might possibly be the precursor of asterubin [dimethylguanidyltaurine, $\text{HN}:\text{C}(\text{NH}\cdot\text{CH}_2\cdot\text{CH}_2\text{SO}_3\text{H})\cdot\text{N}(\text{CH}_3)_2$]. Eight per cent of the substance fed was found in the urine. A conversion, however, into asterubin by methylation could not be assured. [Compare also the work of Ackermann & Heinsen (67)]. Sullivan, Hess & Irreverre (68) studied the excretion of guanidine derivatives in the urine.

Leucine.—Wooley & Petersen (69) analyzed *Aspergillus sydowi* and found that the amino acids leucine and isoleucine are both present, either free or very loosely bound.

Proline.—Das (70) showed that in the kidney of the pig a thermolabile substance, which is to be considered as the activator of proline dehydrogenase, is present. It has also been found in yeast. Its adsorption properties are described. Lactoflavin as well as flavine-enzyme do not seem to participate in this reaction. The same can be said for codehydrogenase I and II.

Krebs (71) showed that the decomposition of proline leads, by way of glutamic acid, to α -ketoglutaric acid. Neber (72) examined the δ -hydroxy- α -aminovaleric acid, prepared by Sørensen, as a possible intermediate product. Surviving slices of liver and kidneys, however, did not convert this acid into the ketoglutaric acid, which

is produced by decomposition of the glutamic acid. The dinitrophenyl-hydrazone obtained was not identical with that of ketoglutaric acid. The author assumes, therefore, that proline is not split by hydrolysis into δ -hydroxy- α -aminovaleric acid, but a direct oxidative opening of the ring must take place.

Hematopoietic substance.—Dakin, Ungley & West (73) studied the hematopoietic substance of liver. They conclude that it must be either a peptide or an association product of a peptide, resembling an albumose. Glucosamine, which was found in former work, is not an essential component of the active principle. The clinical potency of the glucosamine-free peptide is about twice that of earlier products. The hydrolysis of the peptide and the isolation of different amino acids have been described. The peptide has a molecular weight greater than 2000 and less than 5000. Hogan, Guerrant & Ritchie (74) examined deaminized casein for its action in anemia.

Mimosine.—Renz (75) found in the phloem of young shoots of *Mimosa pudica* a substance of the probable formula $C_{16}H_{20}O_8N_4$. It gives with ferric chloride an intensive color reaction and is probably a hydroxyamino acid. This "mimosine" is able to give the stimulus reaction of the pinnula.

Specific dynamic action of amino acids.—In a series of investigations Masai, Mabuchi & Fukiwake (76) studied the specific dynamic action of amino acids. In starvation the action of glycine is weakened; it is increased by glucose. The latter substance is necessary, therefore, for the phenomenon to be observed. Alanine, glutamic acid and aspartic acid exert an effect, but the natural amino acids are stronger than the optical antipodes. In a certain concentration the amino acids show an optimal action.

PROTEINS

Composition and metabolism.—On the question of the constancy in the composition of proteins a lengthy polemic has taken place between Abderhalden & Siebel (77) and Schenk (78). Abderhalden and his students assume that the proteins of a given group of cells must always preserve a constant composition, as otherwise the organism could not dominate the metabolism of the cells. Contrary to this, Schenk assumes that the proteins of the organism must vary in their amino-acid composition according to the metabolic state. The experiments of Abderhalden showed that the plasma- and serum-proteins of the horse, cow, and rabbit which, during four-week periods were

fed with green food, bran or oats, remained unchanged in amino-acid composition.

Abderhalden, Baertich & Ziesecke (79) analyzed the muscle proteins of the cod and found all of the known biologically-active amino acids. In feeding rats with muscle proteins of the cod, as the sole protein component of the diet, it was found that, on the average, the weight increase, during a period of fifty-nine days, was 121 per cent; rats to which casein was administered as the sole protein component only showed an increase of 105 per cent in body weight.

Lissitzin & Alexandrowskaja (80) analyzed the different protamines of the sturgeon. These authors came to the conclusion that there is no difference between the acipenserines of different species of *Acipenser*.

Conner & Sherman (81) found that the protein content of the diet, in respect to its influence on the growth of the rat, is a function of the calcium content also.

Tomita & Kumon (82) examined the larvae of the flesh fly (*Sarcophaga carnaria*). Besides the hexone bases they found proline, phenylalanine, and glutamic acid. Of the proteases, pepsin, trypsin, tyrosinase, nuclease, deamidase, and urease were shown to be present.

Luck (83) reports on the problem of the storage of proteins in the animal organism. For this purpose protein-rich and protein-poor diets were administered to white rats and after two weeks the liver was examined in respect to its protein content. For these experiments a method of protein fractionation was worked out. Four fractions were distinguished: globulin II, euglobulin, pseudoglobulin, and albumin. The blood-free livers were extracted at 0 to 3° in order to exclude the activity of the proteases. It was thereby shown that an enrichment of the liver in proteins extends regularly and proportionally to all of the protein fractions examined. The increase of the different fractions was 50 to 60 per cent. The evidence suggests that none of the liver proteins can be singled out as a reserve, labile, or cell-inclusion protein in the classical sense of the Pflüger-Voit theory to the exclusion of the basal structural proteins of the organ. All of the liver proteins participate equally in the function of storage. The protein contents of muscle, kidney, and intestine are affected relatively little by the changes in diet. Protein enrichment of the liver, following upon the administration of high-protein diets, is associated not only with hyperplasia, or hypertrophy, but with an increased content of protein per unit weight of tissue. In similar investigations

Addis, Poo, Lew & Yuen (84) showed that white rats, after fasting, lose chiefly the protein of the liver and not that of other organs.² The experiments of Luck (83) are relevant to the nutrition experiments with different diets made by Edlbacher & Jucker (58). These authors examined the purine content of rat organs after diets of high- and low-protein content and after purine-free and purine-rich nutrition (cf. page 279).

The problem of the distribution of chemically characterized proteins has been studied from an immunological point of view by Haurowitz & Kraus (85). Rabbits and guinea pigs were passively sensitized by means of injection of an antiserum against arsanil-protein (*p*-phenylarsinic-acid-azo-protein), and later received an adequate dose of arsanil-protein. One hour after this injection the animals were examined and it was found that the antigen, just as in normal animals, was deposited in the organs of the reticulo-endothelial system, chiefly in the liver. Rabbits which were treated with iodoglobulin, likewise showed a deposit of iodoglobulin in the liver.

Bierich, Lang & Rosenbohm (86) examined the serum fractions under the influence of different dyes and report fluctuations after the addition of such substances as isamine-blue, bile acids, etc. This work explains the decrease of albumin-A observed in cancer. In pursuing these experiments, further, they state that in the serum of cancer patients the tryptophane content of the albumin-II fraction is decreased.

Negelein (87) described the isolation of yeast proteins from Lebedew juice, which are the carrier substances of the dehydrases. Eagle & Vickers (88) investigated the entrance of the diazo group into proteins and found that, in addition to histidine and tyrosine, proline, lysine, and arginine also react with the diazo compound.

For metabolic experiments it is of importance to have at hand a micromethod for the determination of nitrogen in tissue slices. Levy (89) described such a method for making nitrogen estimations in tissue slices in quantities of 0.5 to 6.0 μ g. by digestion with sulfuric acid, Nesslerization, and measurement with the Pulfrich photometer. Magnus-Levy (90) examined and described in a detailed manner the

² In continuing these investigations Addis, Poo & Lew (84) observed that every organ has its own characteristics with respect to the rebuilding of protein when casein is given after a period of protein loss. In muscle the changes in the quantity of protein after casein feeding are small; they are greater in kidney and liver.

urinary proteins in three new cases of Bence-Jones albuminuria. They were shown to be identical with former preparations.

PROTEOLYTIC ENZYMES

Waldschmidt-Leitz & Gärtner (91) reported on the enzymatic cleavage of dihydroxypiperazinecarboxylic acids. In an effort to verify the work of Ishiyama (92), Tazawa (93), and Shibata (94) they showed that dihydroxypiperazinediacetic acid and dihydroxypiperazinemonopropionic acid could not be split, either by purified or by crude enzyme solutions. The results of the Japanese investigators are attributed to experimental errors. According to this work all hypotheses which assume an appreciable participation of dihydroxypiperazine rings in the composition of proteins and of specific ring structures as a means of explaining the specificity of proteins must be considered as valueless.

Bergmann & Zervas (95) report on the inactivation of papain by iodine. In pursuing their investigations on the proteolytic enzymes, Bergmann & Ross (96) showed that papain can be separated into two enzymes. They conclude this from the different effects of phenylhydrazine on HCN-papain. One of the enzymes splits benzoylisoglutamine and hippurylamine and is completely suppressed by phenylhydrazine; it is designated papain-peptidase-I. The other enzyme splits albumin peptone and is not inhibited by phenylhydrazine; it is designated papain-peptidase-II. These names are only provisional and may be altered as soon as the specificities of the enzymes become known in detail. Both papain-peptidase-I and papain-peptidase-II participate in the splitting of gelatine.

Cathepsin.—Mystkowski (97) estimated the activity of cathepsin in the embryo. It was found to be small but, on the other hand, in the vitelline sac it is fifteen to twenty times greater. Orechowitsch (98) reported on enzymatic investigations of the tissues of regenerating organs of amphibia and found thereby a strong increase in the tissue protease, particularly of cathepsin.

Other proteolytic enzymes.—Rubel (99) showed that enhancement of glycolysis provokes an increase of proteolysis in the tissues. Normal and malignant tissues behaved alike.

In pursuing former work Duspiva (100) examined the proteases of keratin-devouring larvae of the moths, *Tineola biseliella* and *Galleria mellonella*. *Galleria*, contrary to *Tineola*, cannot digest sheep wool. It was known that the proteases of these insects are able to

utilize keratin as substrate by virtue of a negative redox potential in the intestinal juice.

Kiesel & Roganowa (101) investigated the action of the trypsin-ferment complex on substituted protein. Edestin was substituted with ethoxyl, sulfonyl, and benzoyl groups and subjected to tryptic digestion. The proteinase is not a carboxy-proteinase as its activity does not depend on the free carboxyl groups; on the other hand free amino and hydroxyl groups seem to play a particularly important rôle in the tryptic decomposition of proteins.

Jørgensen (102) showed that the swelling of dough during baking depends on the activity of the proteases. When glutathione is added to bread dough the proteases are activated; this provokes decomposition of the gluten and liquefaction of the dough. Bodansky (103) showed that phosphatases may be activated by amino acids. Mounfield (104) examined the proteolytic enzymes of sprouted wheat.

For the estimation of small quantities of glycine Abderhalden & Neumann (106) utilized the *o*-phthalaldehyde reagent recommended by Zimmermann (107). They showed that this reagent allows the estimation of glycine in pseudoglobulin, whereas albumin and euglobulin give negative reactions. In peptides the reaction is positive only when glycine contains the free amino group. This behavior is of importance for the study of the enzymatic decomposition of peptides.

Schmitz (108) showed that fibrinolysis is a proteolytic process conditioned by a tryptic enzyme which adheres to the fibrin.

MISCELLANEOUS INVESTIGATIONS

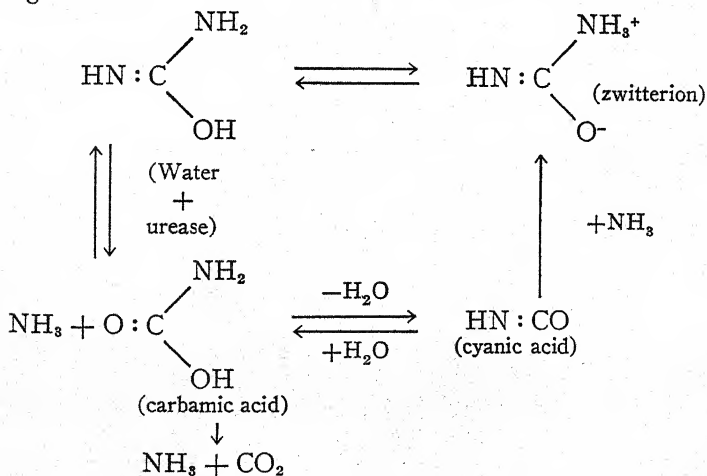
Terroine & Lauresco (109) report the results of studies on digestion and endogenous metabolism. Beard (110) examined the correlation coefficients of various nitrogenous constituents of urine calculated from data obtained by analysis of the urine of 400 normal male medical students. The metabolic relationships existing between total nitrogen and urea, ammonia, uric acid and creatinine are very significant when a diet rich in protein is ingested. These correlations are greater than average analytical results would indicate. The relationship between urea, uric acid, and creatinine, on high-protein diets, indicates a possible exogenous origin of these three substances. A relationship between uric acid and creatinine nitrogen (with one exception) is shown. The rôle of histidine and other amino acids as precursors of these substances is discussed. Hutchinson & Morris (105) studied the nitrogen metabolism of ruminants.

Müller (111) showed that lysine, tyrosine, and glycine are present in the bile as free amino acids; valine, leucine, and other compounds are also present. It is supposed that the organism excretes these amino acids through the bile in the course of conversion of proteins foreign to the species into proteins characteristic of it. The free choline found in the bile is also considered as being a preformed constituent and not a decomposition product of lecithin.

Uric acid.—Edson & Krebs (112) have worked out a new micro-method for the estimation of uric acid: (a) oxidation of the uric acid with manganese dioxide to allantoin; (b) alkaline hydrolysis of the allantoin at pH 10 to allantoic acid; (c) acid hydrolysis of the latter at 100° C., and at a pH of 2, to urea and glyoxylic acid; (d) urea is then estimated manometrically or the glyoxylic acid is estimated colorimetrically or by titration. With this method the errors of the procedures hitherto used are avoided in determining uric acid in organ slices.

Edson, Krebs & Model (113) studied the formation of uric acid in birds. They were able to show that in the first stage of the synthesis ammonia is used in the liver and, with another substance (perhaps lactic acid or pyruvic acid), hypoxanthine is formed; the latter substance could be prepared. In a second stage of the reaction the hypoxanthine is converted in the kidneys (or in the pancreas) by xanthine oxidase into uric acid. [Cf. Schuler & Reindel (114).]

Urea.—Fearon (115) examined the structure of urea in connection with its decomposition and synthesis by urease. He proposes the following scheme:



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DETOXICATION MECHANISMS*

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From the trend of the current biological literature, one can discern the beginning of a realization that the so-called detoxication mechanisms are not isolated biochemical curiosities, but probably constitute vital processes in the body economy. An attempt will therefore be made in this review to interpret the various results, often of highly divergent types of research, as contributions aiding in the solution of more or less concise and circumscribed physiological problems, rather than merely listing results under the well-known and established headings.

The liver and detoxication.—The liver because of its strategic position between the gastro-intestinal system, the chief portal of entry for toxic substances, and the body proper has long been held as an important detoxifying organ, and there is much data to support this view. Further evidence has been secured to show that the production of glucuronic acid is influenced by liver injury. Nishimura (1) reported that camphor failed to cause any increase in the output of glucuronic acid in the dog after total hepatectomy. Persova (2) noted that the rabbit produced much less mentholglucuronide after being poisoned with arsenic. Naturally further attempts have been made to develop liver-function tests on the basis of the output of glucuronic acid. Salt (3) has improved Tollen's naphthoresorcinol test and suggests applying it to urine, collected after the oral administration of acetyl salicylic acid, as a means of showing alteration in the function of the liver. Nasarijanz (4) has continued his studies on the synthesis of mentholglucuronide and found it to be lowered in various liver diseases. One of the greatest difficulties encountered, but often overlooked, is the fact that various amounts of the glucuronogenic drug or aglucone may be destroyed in the body by oxidation. Thus, menthol as well as mentholglucuronide is almost completely destroyed by the dog (Quick, 1928). Pryde & Williams (5), corroborating the earlier work of Quick, found that in man only about 80 per cent of ingested borneol is excreted as borneolglucuronide. Waelsch & Klepetar (6) demonstrated that benzoic acid is oxidized by a preparation

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of ground horse kidney. It follows that the amount of conjugated glucuronic acid excreted is not always a true measure of the quantity synthesized but rather of the amount which has escaped oxidation, and it is conceivable that in certain instances an increased excretion may actually be pathological. It is interesting that drugs which are presumably detoxified by the liver also exert an effect on this organ. Kobayashi (7) found that tribromoethyl alcohol in the dog caused a marked increase in the volume of the bile, but at the same time a pronounced decrease in the output of bile acids. In the rabbit the choleretic effect was not observed, but a decrease in the glycogen of the liver was noted. The author concludes that the drug damages the liver. In such experiments it is probably best to differentiate between actual liver damage and temporary inhibition of function. Nakagawa (8) also observed that tribromoethyl alcohol and chloral caused an increase in the flow of bile but with a depression of oxygen consumption in contrast to other choleretic drugs such as cinchophen and euphyllin, which increase the rate of oxidation. Okii (9) found that if bile acids were given to a rabbit before the administration of phenol, the output of ethereal sulfates was increased. Okii postulates that bile salts increase the detoxifying power of the liver by increasing the glycogen supply. Tsutsui's (10) results emphasize the fact that the detoxication of drugs is not entirely dependent upon the liver, for he found that the minimum lethal doses of the three isomeric hydroxybenzoic acids are essentially the same in hepatectomized as in normal dogs.

The utilization of the hippuric acid synthesis as a test of liver function is receiving clinical consideration. On the basis that the glycine required for the conjugation is produced by the liver, Quick (11) concluded that the production of hippuric acid could be used as a test of liver function. The study was facilitated by the development of a simple quantitative method for determining hippuric acid. Quick (12) found that the normal output of benzoic acid, combined as hippuric acid, in four hours following the ingestion of 6 gm. of sodium benzoate is approximately 3 gm. In various types of liver disease the output is diminished and often the decrease corresponds well with the gravity of the condition. In uncomplicated cholecystitis the test is normal, suggesting the absence of serious intrahepatic damage. Vaccaro (13) and Snell & Plunkett (14) have corroborated these findings and have found a reasonable agreement between the test and the degree of pathology morphologically observed in the liver. Kohl-

staedt & Helmer (15) likewise found the synthesis of hippuric acid lowered in patients with damaged livers. Adlersberg & Minibeck (16) have studied the synthesis of hippuric acid in rabbits after producing liver injury with phosphorus and with carbon tetrachloride and have found a diminished production. Kanzaki (1933) likewise observed a diminished synthesis of hippuric acid in rabbits poisoned by these agents. Adlersberg & Minibeck (17) have observed that the output of hippuric acid is low in humans with damaged livers, but they feel that the diagnostic value of the test is impaired by the fact that the hippuric acid output is also low in mechanical obstruction of the biliary passage, in severe cardiac decompensation, and in cachectic conditions. They apparently do not consider that in such conditions the function of the liver may be impaired. Bartels (18) has applied the test to cases of hyperthyroidism and has made the interesting observation that the test runs fairly parallel with the severity of the condition, especially as judged by the loss of weight; furthermore, that as the patient improves under the administration of iodine or by partial thyroidectomy the output of hippuric acid is greatly increased. Thus the test tends to confirm the view that impairment of liver function is present in thyrotoxicosis.

Much remains to be learned concerning the metabolism of benzoic acid. Thus, the toxicity of this compound seems to be influenced by various salts. Lombroso, Zummo & Stassi (19) found that the toxic action which manifests itself when benzoic acid is added to a diet just adequate to maintain normal growth of guinea pigs could be counteracted by the sodium salts of citric, tartaric, and glycocholic acids. Griffith (20) observed that even simple inorganic sodium salts acted efficiently and, in a later study (21), showed that the action was not due to any effect on the kidney.

Uric acid.—The existence of a relationship between the detoxication mechanisms and uric acid metabolism is definitely suggested by a number of observations. Lewis & Karr (22) were the first to note that benzoic acid depresses the excretion of uric acid. Quick (23) observed that phenylacetic acid and other aromatic acids likewise suppress the output of uric acid. He further noted that various compounds such as pyruvic acid and glucogenetic amino acids which stimulate glucuronic acid production also increase the rate of uric acid excretion, whereas substances such as lactic acid and acetoacetic acid which reduce the formation of glucuronic acid also diminish the excretion of uric acid. Quick (24) has further found that the de-

pressing action of benzoic acid on uric acid output is greatly intensified in certain types of liver disease. An interrelation of uric acid metabolism, detoxication mechanisms, and the liver is thus indicated and this may perhaps serve as a lead in the elucidation of the problems of purine metabolism.

Glucuronic acid.—This uronic acid has a unique place in the detoxication systems of the body, for not only can it be produced in relatively large amounts but it can also be combined in two modes of linkage, the glucoside or glucuronide, and the ester type. Disagreement concerning the structure of the latter has arisen. Pryde & Williams (25) disagree with Quick that the compound formed by the conjugation of glucuronic acid with benzoic acid is a glucuronic acid monobenzoate.¹ They claim that it is a benzoyl glucuronic acid, for their experimental findings suggest that potassium cyanide does not act directly on the conjugated compound but first causes hydrolysis and then reacts with the liberated glucuronic acid. Quick (26) defends his structure by showing that mutarotation occurs without any evidence of hydrolysis, and that when the cyanohydrin reaction is 96 per cent complete only 8.9 per cent of the compound has hydrolyzed. Masamune (27) supports the monobenzoate structure, for he likewise has observed mutarotation in alkaline solution without the occurrence of hydrolysis. The finding that the aldehyde group of glucuronic acid in the ester type is free becomes of importance in considering the formation of glucuronic acid in the body. Thus, the theory of Fischer & Piloty that in the formation of conjugated glucuronic acid an intermediary glucoside is formed which protects the labile aldehyde radical from oxidation, loses its significance. That this theory is untenable is further shown by Pryde & Williams (28) who found that phenyl α - and β -glucosides are not oxidized to the corresponding glucuronides in the organism, but undergo complete hydrolysis whereupon the liberated phenol is conjugated with sulfuric acid to the same extent as an equivalent amount of phenol administered directly.

The hydrolysis of glucuronides apparently requires an enzyme different from that which splits β -glucosides. Bergmann (29) found that emulsin did not hydrolyze β -naphthol glucuronide. Helferich & Sparmberg (30) believe that the glucuronides require a special ferment, although they obtained evidence that emulsin does hydrolyze mentholglucuronide. Masamune (27, 31) and Oshima (32) have

¹ Cf. *Ann. Rev. Biochem.*, 2, 387 (1933).

studied the action of an enzyme extracted from ground kidney which readily hydrolyzes conjugated glucuronic acids. This enzyme which they named β -glucuronosidase is widely distributed in the body; little is found in the blood, but it is interesting that a high concentration is present in the spleen and endocrine organs.

The position of glucuronic acid in carbohydrate metabolism is still undetermined. Enklewitz & Lasker (33) have made the significant observation that ingestion either of glucuronic acid or of various glucuronogenic drugs greatly increases the output of *l*-xyloketose in pentosuric subjects while the feeding of conjugated glucuronides does not. This suggests that the common urinary pentose, *l*-xyloketose, originates from an aberrant metabolism of glucuronic acid. Mucin did not increase the pentose production which speaks against the assumption that this substance is an important source of glucuronic acid for the purpose of conjugation. Even if it were, the question remains as to where and how the body obtains the glucuronic acid for the synthesis of mucin. It seems that the protective action of mucin and of pectin (34) must be looked for elsewhere than in their content of uronic acids.

The number of compounds found to be conjugated with glucuronic acid is constantly increasing. Enklewitz (35) demonstrated that the reducing substance found in the urine after the ingestion of amidopyrine is a conjugated glucuronic acid complex but the compound was not isolated. Tsunoo (36) studied the fate of the drug, ethynal (furylacryl-*p*-hydroxyphenylurea), and found that the body hydrolyzes it to furylacrylic acid, which it conjugates with glycine, and *p*-hydroxyphenylurea which it combines with glucuronic acid. Horn (37) reported that dimethylaniline in the rabbit is converted to *p*-(mono)methylaminophenol and is excreted in combination with glucuronic acid. In carnivora such as the dog (38) the same compound is changed to *o*-aminophenol. In this animal methemoglobin is formed, but not in the rabbit, showing that the detoxication mechanisms do modify toxic actions.

Hormones.—Recent work on the sex hormones suggests the interesting possibility that the detoxication mechanisms may perhaps play an important part in the control of these agents. While it was recognized that the oestrogenic substances in pregnancy urine may be present in a combined or inactive form, nothing was known of the chemical nature of these inactive substances. Cohen & Marrian (39) have now succeeded in isolating a compound, the elementary compo-

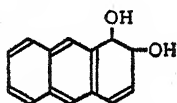
sition of which corresponds to oestriol glucuronic acid. That the compound is a glucuronide is indicated by the fact that it reduces Benedict solution only after hydrolysis and that it gives a positive reaction with naphthoresorcinol. Further study has shown that the glucuronic acid is not conjugated with the phenolic hydroxyl group (40). Curiously, the excretion of free or active hormone in the human greatly increases at term (41). The earlier observations of Cohen & Marrian (42) suggest that this hormone may be combined in a form other than a glucuronide, for on heating pregnancy urine with sodium hydroxide 50 per cent of the combined form was hydrolyzed. Since the common glucuronides are resistant to alkaline hydrolysis the possibility of a second type of conjugation is suggested. Schachter & Marrian (43) have now found that the combined oestrogenic substance in mares' urine is not a glucuronide but appears to be a sulfate. Odell & Marrian (44) have noted that a large fraction of pregnandiol in human pregnancy urine is present in a combined form. Venning & Brown (45) have isolated pregnandiol glucuronide. The work of Adler (46) suggests that a portion of the androgenic substance, the male hormone, also appears to be present in the urine as a conjugated and inactive compound.

Oxidation.—The destruction of noxious substances by oxidation constitutes one of the most important detoxication mechanisms, and the identification of oxidative degradation products has been useful in securing useful data on the chemistry of metabolism. The significant work of Verkade and of Flaschenträger and their associates on ω -oxidation has been discussed in previous reviews. Kuhn, Köhler & Köhler (47) studied the oxidation of geraniol in the dog. They found that one of the end methyl radicals is oxidized to a carboxyl group. While they recognize that this reaction may be looked upon as an ω -oxidation they consider that it belongs to a more comprehensive category, namely, methyl oxidation. The methyl group need not necessarily be an end group for Asahina & Ishidate (48) have shown that the body oxidizes the methyl group attached to the central carbon atom of the camphor molecule. The interesting suggestion is made by Kuhn *et al.* that perhaps ω -oxidation is one form of methyl oxidation.

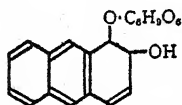
Researches on the oxidation of various substances in the presence of sliced organs have yielded a variety of results. Jowett & Quastel (49) found that benzoates, cinnamates, and phenylpropionates strongly inhibit the oxidation of butyric acid by liver tissue. Mazza

(50) found that phenylbutyric acid and α : β -phenylcrotonic acid, but not β : γ -phenylcrotonic acid, were oxidized in the presence of liver slices. The inability of the liver to shift double bonds seems strange since no such limitation is observed when the acids are metabolized by the whole organism (Quick, 1928). The work of Snapper & Grünbaum (51) demonstrated that the kidney possesses both the power to degradate phenyl aliphatic acids by β -oxidation and to conjugate the end products with glycine. The possibility that the oxidative processes may differ in the growing state from the mature state is suggested by Cerecedo & Stekol (52) who report that isobarbituric acid in the growing dog is not metabolized to urea as in the adult animal.

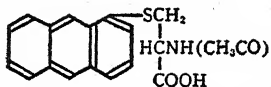
Polycyclic hydrocarbons.—The fact that the sex hormones and certain carcinogenic compounds are derivatives of polycyclic hydrocarbons has stimulated studies on the fate of aromatic hydrocarbons in the animal body. These endeavors serve as a delightful contrast to those desk-born hypotheses that aim to explain the toxic action of a series of different drugs on the basis that they all possess a benzene ring. The investigation of Boyland & Levi (53) on the metabolism of anthracene is an outstanding contribution. They were able to isolate and determine the structure of three derivatives of anthracene formed in the body:



I. 1,2-Dihydroxy-1,2-dihydroanthracene



II. 1,2-Dihydroxy-1,2-dihydroanthracene glucuronide



III. 1-Anthrylmercapturic acid

The authors believe that it is possible that some 9,10-anthraquinone and a hydroxy dihydroanthracene are also formed. Curiously, the rabbit excretes the *d*-form of I, while the rat produces the *l*-form. Boyland & Levi do not commit themselves on the question whether these compounds represent successive steps in the metabolism of anthracene or whether they are formed independently of each other.

Similar studies on 1,2,5,6-dibenzanthracene, the well-known car-

cinogenic hydrocarbon, are still lacking, but Berenblum & Kendal (54) have shown that it can be destroyed by the mouse. It is generally recognized that a relationship exists between malignancy and sulfur metabolism, and also that a number of polycyclic hydrocarbons are carcinogenic. May it be that the conjugation of these hydrocarbons with sulfur (as mercapturic acids) is a connecting link between the two factors? It is rather curious that Reimann & Hall (55), contrary to their expectations, found that the sulfhydryl-containing compound, *p*-thiocresol, markedly diminished the incidence of carcinoma in the mouse induced by 1,2,5,6-dibenzanthracene. Connor, Carr & Ginzton (56) furthermore report the interesting observation that injection of cysteine hydrochloride into a Jensen's sarcoma caused necrosis and tumor regression.

Mercapturic acid.—With the realization that the study of the synthesis of mercapturic acids may be a valuable approach to new knowledge concerning sulfur metabolism, intensive work on the fate of bromobenzene and naphthalene has been continued, especially by Stekol. Stekol (57) has devised a quantitative method for determining *p*-bromophenylmercapturic acid which is based on the formation of *p*-bromophenylmercaptan from mercapturic acid by alkaline hydrolysis. This derivative can be precipitated as a mercury salt or determined iodimetrically. Stekol has found that growing dogs and mice can readily form *p*-bromophenylmercapturic acid when fed an adequate diet (58), but even after a twenty-eight to thirty-five day fast this synthetic ability is not lost in the dog (59). In other words, the organism will supply cysteine to detoxicate bromobenzene at the expense of its own tissues if no exogenous cystine or methionine is available. On this basis White & Jackson (60) have utilized bromobenzene in growth experiments in which they found that when this substance was incorporated in an otherwise adequate diet, the growth of white rats was retarded. Significantly, the addition of either cystine or methionine readily overcame this retarding influence whereas taurine, sodium sulfate, and digested casein were ineffective. The authors conclude that bromobenzene brings about its growth-retarding action by combining with the two sulfur amino acids and thus depriving the body of these compounds which are essential for growth. Stekol (61) has shown that when naphthalene is fed to dogs a portion is excreted as the mercapturic acid, which he actually isolated, while another fraction is apparently oxidized to a naphthol and excreted in combined form, partly with sulfuric acid and partly with glucuronic

acid. It is quite probable that with suitable methods it will be possible to show that perhaps all mammals can synthesize mercapturic acids. Stekol (62) demonstrated that the pig possesses this synthetic ability. Curiously, this animal seems to have a very limited capacity to produce ethereal sulfates as tested by isobarbituric acid. Virtue (63) showed that the cat synthesizes *p*-bromophenylmercapturic acid even at the expense of its own tissues. In general it seems that aromatic hydrocarbons lacking aliphatic side chains are in part changed to the mercapturic acid. As already mentioned, Boyland & Levi (53) isolated anthrylmercapturic acid, and Stekol (64), in agreement with Callow & Hele (1926), has evidence that benzene and phenanthracene are likewise conjugated.

From these results one is led to speculate whether the union of cysteine with the hydrocarbon does not represent the first and perhaps only step available to the organism for attacking the unsubstituted aromatic ring, and that as a further reaction the mercapturic acid is replaced by a hydroxyl group. The organism can apparently utilize both organic and inorganic sulfur for the production of ethereal sulfates as Stekol and earlier workers have shown, but the cysteine required for mercapturic acid must come either from the two sulfur amino acids in the diet or be derived from the breakdown of tissues; it is this fact that perhaps partially accounts for the subtle toxicity of benzene and the polycyclic hydrocarbons.

Cataract.—It has long been known that cataracts can be produced by feeding naphthalene. Nakashima (65) found that the incidence of cataract in rabbits from naphthalene could be greatly decreased by feeding cystine, but even sodium sulfate seemed to have a protective action. In agreement with the observations of earlier workers he found that derivatives of naphthalene, such as the naphthols, did not cause lenticular opacity which he believes is due to the fact that these derivatives can be combined with glucuronic acid whereas naphthalene unites with cysteine. Nakashima also found a reduction of the glutathione content of the eye. Tsuji (66) previously had shown that the cysteine content of the lens was decreased after a cataract had been induced by naphthalene. In senile cataract a reduction in glutathione also appears to occur as von Euler & Martius (67) and others have observed. It appears that a disturbance of the oxidation-reduction system, such as can be brought about by a decrease of glutathione, results in opacity of the lens. It must be remembered that ascorbic acid is also present in the eye and appears to act as a

second redox system but curiously, even though the content of ascorbic acid can be reduced to a mere trace in experimental scurvy, neither Biette & Carteni (68) nor Johnson (69) noted any changes in the lens. Significantly, Smaltino (70) found the glutathione content of the eye was normal in guinea-pig scurvy. Perhaps the work of Hopkins & Morgan (71) supplies the answer. They observed that when glutathione and ascorbic acid are together in the presence of hexoxidase, the former protects ascorbic acid from oxidation and acts as if it alone were present in the system. So it appears that as long as glutathione, with its predominating influence, is present in the eye the lack of vitamin C is of little moment. Dinitrophenol also causes cataract. Cogan & Cogan (72) believe that this is due to tissue anoxemia which damages the lens epithelium. Josephson (73) brought about great improvement in the lens by giving patients ascorbic acid but, incidentally, he supplemented this treatment with a diet rich in glutathione. Evans (74) reported that in the cataract resulting from thyroparathyroidectomy the glutathione content is reduced, thus suggesting a possible influence of calcium in the detoxication mechanisms.

Phenylacetyl glutamine.—The conjugation of phenylacetic acid with glutamine has hitherto been observed only in man but Power (75) has now shown that the chimpanzee likewise possesses this synthetic process.

Vitamins.—Little effort has been made to determine whether lack of vitamins influences the detoxication mechanisms. Kumon (76) reports that no marked impairment of the conjugation mechanisms occurs in avitaminosis-B; the excretion of benzoic acid seems somewhat delayed and there is a slight inhibition of ethereal sulfate formation.

Mandelic acid.—An interesting illustration of the relation of the detoxication processes to therapeutic activity is the behavior of mandelic acid in the body. Rosenheim (77) employed the acid as a substitute for ketosis to control infection of the urinary tract. Helmholz & Osterberg (78) have further studied the excretion and bactericidal power of this compound. The recent findings that the acid is excreted uncombined was noted by Schotten in 1883. The excretion of free mandelic acid is explained by Quick (1932) on the basis of his theory that one function of conjugation is the conversion of a weak acid to a stronger one so that it can be more readily excreted. Since mandelic acid is a relatively strong acid ($C_H = 4.3 \times 10^{-4}$) it does not require conjugation for its elimination.

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THE HORMONES*

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The vast number of papers published during the past year upon the many aspects of endocrinology has rendered impossible the task of condensing a comprehensive review of the hormones into the space allotted. Accordingly the authors have confined their attention to a few topics which appeared to them to be ripe for review, and they have endeavoured to treat them in a comprehensive and critical manner. They wish, therefore, to tender their apologies to the many authors whose papers have not been reviewed. In the belief that the function of reviewers is to review, the authors have not hesitated to express freely their own opinions on certain topics. To avoid possible misunderstanding they have attempted to indicate clearly the places where this has been done.

THE ADRENAL CORTEX

Only a few years ago, the one fact known with certainty regarding the adrenal cortex was that it was essential for life. Recent work, much of which has been carried out during the past year, suggests that this gland may occupy a position in the whole endocrine system second only in importance to that of the pituitary itself. While the outstanding problem of the exact chemical nature of the "life-maintaining" hormone" has at the time of writing not been solved, our knowledge concerning the mode of action of this hormone is rapidly being clarified, and it is becoming increasingly probable that the gland may have many functions other than that of elaborating this principle.

The chemistry of the adrenal cortex.—Researches from three different laboratories directed toward the isolation of the life-maintaining hormone have shown that from the cortex may be isolated a number of apparently closely related, hitherto unknown, crystalline substances, some of which are ketonic in nature, and many of which have twenty-one carbon atoms (1-12). One of the compounds isolated by Reichstein (7) is an $\alpha:\beta$ -unsaturated diketone of the probable formula $C_{19}H_{24-26}O_3$. This substance, adrenosterone, has marked androgenic activity by the capon-comb test. Reichstein (8) and Ken-

* Received December 28, 1936.

dall and his coworkers (4, 5) have degraded certain of their C_{21} compounds to substances which also have androgenic properties. These findings are of particular interest in view of the frequent association of virilism in women with hypertrophy or tumour of the adrenal cortex (cf. Broster & Vines, 13). In this connection it is interesting to note that an excess of androgenic hormone has been found to be present in the urine of virilism cases by Simpson (14), and more recently by Simpson, deFremery & Macbeth (15) and Gallagher (16). Callow (17) has recently reported the isolation of the androgenic substance from the urine of a patient with an adrenal tumour. At the time of writing, however, no details of this work were available to the reviewers. The androgenic potency of the compounds isolated from the cortex led Reichstein (7, 8) and afterwards Mason, Myers & Kendall (3) to suggest that the compounds in question had a steroid structure. Reichstein (9) has now provided strict chemical proof for the presence of the androstane skeleton in certain of the compounds.

Space does not permit of a fuller description of the chemical nature of this interesting new series of steroid compounds, but one of them must be dealt with at greater length in another connection. Kendall *et al.* (3) isolated from adrenal extracts a substance [m.p. 201–208°; $[\alpha]_{5461} = +269^\circ$] which appeared to have the formula $C_{21}H_{30}O_5$. Since it showed an absorption band at 2370 Å it was believed to be an α : β -unsaturated ketone. In later papers (4, 5) the formula $C_{21}H_{28}O_3$ was preferred and a structural formula, based on its probable but then unproved steroid nature, was suggested. Cortin-like activity was detected on testing this compound by the method of Ingle (18). The potency of this substance was stated to be "much less than that of cortin itself" and the authors did not exclude the possibility that the activity of the crystallizate was due to contamination with traces of the hormone. Since, however, the potency remained unchanged after crystallization from several solvents, they regarded this possibility as remote. Wintersteiner & Pfiffner (12) and Reichstein (10) have reported the isolation of what is almost certainly the same compound. The former report that it is inactive when tested on the adrenalectomized dog, and the latter reports that it is inactive by the Everse-deFremery (19) rat test. In the case of the oestrogenic, androgenic, and progestational hormones, physiological activity is shown by more than one compound in each class. It would not be surprising, therefore, if cortin-like activity proved to be a property of more than one substance. However, in view of the differ-

ence of opinion concerning the activity of the substance in question, a final decision must await further results.

In this connection the reviewers wish to point out that in their opinion the only reliable criterion of cortin activity is the maintenance of life in adrenalectomized animals such as the cat and dog, since life maintenance is still the only function of this hormone upon which there is complete unanimity. The principle underlying both the Ingle and the Everse-deFremery tests is that after adrenalectomy the muscles of rats are more prone to fatigue. The administration of cortical extracts to the rats restores the normal work capacity of the muscles. Lang & Stuber (20) have recently made the disturbing statement that the principle in adrenal extracts which increases the work output of the isolated frog gastrocnemius is methyl stearate or methyl palmitate. In view of this finding it would appear that the methods of assay based on the fatigue of rat muscle should be used with caution. Furthermore it should be emphasized that methods of assay based on growth changes, resistance to toxic substances, regulation of body temperature, blood-urea changes, etc., should also be used with caution. Until the life-maintaining hormone has been isolated in a state of chemical purity, it is impossible to ascertain which of the symptoms of adrenal insufficiency are specifically due to a lack of this principle.

The physiological rôle of the adrenal cortex.—Mainly as a result of the well-known work of Loeb, Harrop and their respective co-workers, the view has been widely accepted that cortin has as its principal function the regulation of the sodium and water content of the blood through its influence on the renal excretion of these substances. During the past year this view has been challenged in important papers published from several laboratories.

It is a well-established fact that animals suffering from adrenal insufficiency show a high blood potassium which may be corrected by the administration of cortin. Harrop and his coworkers (21) state that the blood-potassium level may be brought back to normal by the administration of sodium chloride alone provided that the insufficiency symptoms are not too acute. They regard the elevation of the blood potassium as part of the mechanism by which the osmotic changes resulting from the high renal excretion of sodium chloride are dampened. The high toxicity of potassium salts to adrenal insufficient animals has been shown by Allers *et al.* (22), Zwemer & Truszkowski (23), and Cleghorn & McVicar (24). The results

strongly suggest that the potassium content of the test animals' diet is an important and hitherto much neglected factor in the assay of cortin concentrates. Zwemer & Truszkowski (23, 25) have shown that the muscle potassium is but slightly lowered in adrenalectomized animals. From this they argue that both liberation of intracellular potassium and renal retention are factors concerned in the rise of blood potassium. They have shown that there is a striking parallelism between the effects of sublethal doses of potassium and the symptoms of cortical insufficiency. Cats in which the blood potassium was maintained at a level of 30 mg. per cent for several hours showed anorexia and severe muscular asthenia. They quote other authorities to suggest that the low blood sugar, dehydration, sodium loss, and kidney damage, seen in cortical insufficiency may be secondary to the primary defect of a disturbance in potassium metabolism.

Silvette & Britton (26, 27) have reported that adrenalectomy in the opossum and the marmot results in a rise, rather than a fall, in blood sodium chloride. They have also been able to demonstrate a marked retention of ingested sodium chloride and water by the adrenalectomized opossum (26, 28). They state (26):

It is hardly likely that opposite shifts in sodium and chloride and water balance would be observed after adrenalectomy in different animals if the cortico-adrenal hormone were specifically related to salt and water regulation.

It is, however, a puzzling fact that the life of adrenalectomized opossums may be prolonged by saline administration (29).

Swingle *et al.* (30) observed that the symptoms of acute adrenal insufficiency in the dog could be relieved by the administration of cortin even though food and water were withheld from the animals. Since the alleviation of the symptoms was accompanied by a definite dilution of the blood, it was inferred that during the insufficiency fluid was immobilized within the tissues. Swingle *et al.* (31, 32, 33) have now extended this work. They have shown that adrenalectomized dogs fed on diets low in sodium chloride and provided with unlimited water may be maintained in a healthy condition for long periods by cortin administration without any marked rise in the blood sodium chloride above the pre-injection level. It was therefore inferred that the re-hydration of the blood effected by the cortin was a process occurring independently of any changes in the sodium chloride distribution. The experimental depletion of sodium chloride in adrenalectomized dogs by intraperitoneal injections of isotonic glucose

solution by the method of Darrow & Yannet (34) caused immediate collapse owing to the rapid withdrawal of fluid from the blood to the tissues. The administration of large amounts of cortin caused reversal of the fluid shift and immediate recovery, but the blood sodium chloride remained low. Intact dogs similarly depleted of sodium chloride showed a low blood sodium, a low blood pressure, and a high hemoconcentration, but they did not collapse. Cortin administration restored the blood pressure and concentration to normal but the blood sodium chloride remained low provided the animals were maintained on a salt-free diet. In passing, it may be noted that these authors suggest the use of intact sodium-chloride-depleted animals for cortin assay. Swingle and his school therefore believe that the essential cause of the adrenal insufficiency syndrome is the withdrawal of water from the blood to the tissues. They do not believe that the high hemoconcentration is essentially the result of increased excretion of salt and water by the kidney. That the tissues of animals suffering from adrenal insufficiency are hydrated is not accepted by all authorities [cf. Grollman (35) p. 183]. Truszkowski & Zwemer (25) have, however, recently reported figures which clearly indicate that in the cat such a hydration may occur. It is interesting at this point to note that Thorn *et al.* (36) have shown that large doses of cortin administered to normal humans will cause a reduction of sodium excretion and an increase in potassium excretion.

Britton and his school have long maintained that the primary defect in adrenal insufficiency is an interference with normal carbohydrate metabolism. They have recently pointed out (26) that

all mammalian types which have been observed—including the dog, cat, rabbit, guinea-pig, rat, opossum and marmot—show unidirectional shifts in carbohydrate values after adrenalectomy: blood glucose and hepatic glycogen are reduced to levels incompatible with life, and muscle glycogen is notably decreased.

Harrop *et al.* (21) suggested that the carbohydrate changes observed after adrenalectomy were secondary effects resulting from the inanition accompanying the insufficiency. Silvette & Britton (26) in meeting this criticism have pointed out that cats may show definite symptoms of insufficiency together with the characteristic changes at a time when inanition could not be a serious factor. Furthermore, they point out that cats starved for twelve days show far less pronounced changes in carbohydrate (blood sugar and hepatic glycogen) than are observed in adrenal insufficient cats. Some support for Britton's main thesis is provided by the work of Buell, Anderson &

Strauss (37) who showed that adrenalectomized rats, even though maintained in good health by salt administration, had a lowered power to convert lactic acid into glycogen in the liver.

Work carried out during the past few years in two European laboratories strongly suggests that the primary defect in cortical insufficiency is much more deep-seated than has been supposed by the previously mentioned authors. In a long series of papers, Verzář and his coworkers (38-42, 43,¹ 44¹) have traced a most impressive parallel between the effects of cortical insufficiency in rats and poisoning with iodoacetic acid. In the opinion of the reviewers this work does not seem to have received the widespread recognition that it merits. Both cortical deficiency and iodoacetate poisoning were found to result in a slowing of fat absorption from the intestine and abolition of the selective absorption of glucose. These effects are ascribed to the failure of the mechanism for the phosphorylation of the fat and the glucose which is a necessary preliminary to their absorption. It was also found that phosphorus poisoning in animals deprived of their adrenals or poisoned with iodoacetate did not lead to the production of fatty livers. It is argued, therefore, that in both conditions there is a failure of phosphorylation of the fat which is a necessary preliminary to its mobilization from depôts to the liver. The hypoglycemia which is observed in adrenal insufficiency, particularly after muscular work, is ascribed to a failure of the normal restitution processes in the muscles consequent upon the interference with the phosphorylation process. The growth failure observed in both conditions is ascribed to the failure of the organism to phosphorylate dietary flavine, which results in a secondary avitaminosis. In support of this contention it was found that not only cortin administration but also feeding flavine phosphate would cause resumption of growth in adrenalectomized animals or in animals poisoned with iodoacetate. It was furthermore found that cortin would not maintain adrenalectomized animals fed on a flavine-free diet. It is therefore concluded that (44) *"Die Bedeutung des Rindenhormons für den ganzen Körper ist also, dass es des Entstehen der Flavinphosphorsäure des gelben Atmungsfermentes im Körper, möglich macht."* While many may be unwilling to accept this conclusion, it is one that is difficult to avoid in view of the experimental findings. Of particular interest are some further experiments (43) which throw a new light on the mechanism of the

¹ A review of earlier work is included.

blood-sodium loss and redistribution of water that is seen in adrenal insufficiency: Glucose solution was injected into ligatured loops of the intestines of rats poisoned with iodoacetate or suffering from adrenal insufficiency; it was found that sodium salts diffused out into the intestine causing a hypertonic state which then resulted in the diffusion of large amounts of water into the intestine.

The ideas advanced, in what can only be described as a brilliant paper, by Jimenez-Diaz (45) have much, but not everything, in common with those of Verzář. In this paper Jimenez-Diaz reviews critically the experimental results obtained in his own school and elsewhere, and upholds the view frequently advanced by others that many of the symptoms of cortical deficiency are the result of impaired kidney function. Arguing from the facts that Addison's disease is accompanied by a lowered alkali reserve in the plasma and a decreased ammonia excretion, he suggests that the cortical deficiency renders the kidney asthenic and incapable of producing ammonia. This, it is argued, leads to an increased sodium excretion for the maintenance of the acid-base balance, which results in the lowering of the blood sodium. In support of this contention experiments showing that the isolated kidneys of adrenalectomized cats have a lowered power of ammonia production are described. Jimenez-Diaz accepts Verzář's view of the similarity between the effects on the organism of cortin deficiency and iodoacetate poisoning. He has provided support for this view by showing that the phosphatase activity of kidney tissue from adrenalectomized cats is very markedly lowered.

It appears to the reviewers that, on the basis of the ideas presented by Verzář and by Jimenez-Diaz, many of the seemingly irreconcilable facts and theories advanced by the different American schools may be brought into harmony with one another.

The relationship between the adrenal cortex and the gonad-pituitary system.—That hyperactivity of the ovary may prolong the life of certain adrenalectomized animals is a fact that has been recognized for many years. As long ago as 1927 Rogoff & Stewart (46) showed that pregnancy considerably lengthened the survival period of adrenalectomized dogs, and, since the life prolongation was observed in animals which whelped immediately after the operation, it was clear that the foetal adrenals could not have been responsible. These authors (46, 47) also noted a life-prolonging effect of oestrus on dogs. It is interesting to note that Corey (48) showed that neither pregnancy nor lactation prolonged the life of adrenalectomized cats.

More recently Wilder (49) has reported that commercial anterior pituitary extracts exert a beneficial effect in Addison's disease. The same preparation used by Swingle *et al.* (50) was shown to prolong the life of adrenalectomized cats of both sexes. These authors were also able to prolong the life of adrenalectomized dogs of both sexes by the administration of gonadotropic pituitary and urine extracts. Somewhat different results have been reported by Emery & Schwabe (51). They have reported a definite prolongation of the life and an improvement in growth of female adrenalectomized rats given rat-pituitary implants. It is clear that the effect of the pituitary implants was an indirect one caused by ovarian stimulation, since under similar conditions no prolongation of life was observed with ovariectomized adrenalectomized female rats and with adrenalectomized males. It is apparent from this and the preceding evidence that the ovary can, under certain circumstances, elaborate a hormone that has a somewhat similar action to that of cortin. Emery & Schwabe found that 5 rat units of oestrone daily did not prolong the life of the adrenalectomized female rat, so that it would seem unlikely that the substance in question could be one of the oestrogenic hormones. It is also clear from these results that the testis of the cat and dog, but not of the rat, can also liberate a life-prolonging principle. Until further experiments on the possible life-maintaining action of all the known oestrogenic and androgenic hormones and progesterone have been carried out, it would clearly be useless to speculate at length on the possible nature of these cortin-like substances elaborated by the gonads.

The presence in the adrenal of compounds possessing androgenic potency has already been mentioned. The theory that the adrenal cortex exercises a direct control over the male accessory sex organs has recently received support from the work of Davidson & Moon (52). They have shown that treatment of castrated immature male rats with pituitary gonadotropic extract containing the adrenotropic and lactogenic factors, but no growth, thyrotropic or gonadotropic hormones, resulted in not only hypertrophy of the adrenal cortex but also considerable stimulation of the prostate gland. However, little evidence of stimulation of the seminal vesicles was observed. The relationship of the adrenals to the reproductive system has been still further complicated by the findings of Callow & Parkes (53) that horse adrenals contain considerable amounts of a progestational principle and detectable amounts of an oestrogenic hormone. The chemical identities of this adrenal progestin and oestrogen have not

yet been established, although, since a portion at least of the latter is phenolic in character, its identity with certain "oestrogenic" androgens seems to be excluded. In this connection it is interesting to note that Cahill *et al.* (54) have recently demonstrated that in cases of adrenal virilism in women abnormally large amounts of an oestrogenic hormone are excreted.

It now seems probable that the adrenals of several species contain a gonadotropic principle. Casida & Hellbaum (55) showed that extracts prepared from horse adrenals caused ovulation and corpora lutea formation in the immature rat. The possibility that the activity was mainly due to residual blood in the glands was excluded. Confirmation of this work was provided by Deanesly (56). Allen & Bourne (57) were able to obtain extensive luteinization of the ovaries of young rats with trichloroacetic acid extracts of ox and kangaroo adrenals. The principle in the extract was not ether-soluble and from the method of preparation it would appear not to be a protein. Ehrenstein & Britton (58) have also obtained from ox adrenals a gonadotropic preparation. Until experiments with such extracts have been carried out on hypophysectomized animals, it is hardly possible to say whether the observed effects are due to a direct gonadotropic action or to a stimulation of the pituitary, such as may be produced by administration of oestrogenic hormones (see next section). If the action is an indirect one the methods of preparation used would seem to exclude the possibility of a free oestrogen being the pituitary-stimulating agent. However, Ellison & Burch (59) have recently shown that emmenin (oestriol glucuronide?) will cause pituitary stimulation and secondary ovarian effects. Since the adrenal undoubtedly contains oestrogens, it is not impossible that the gonadotropic action of adrenal extracts is due to the presence of a similar water-soluble, ether-insoluble conjugated oestrogen. Somewhat different results have been reported by Hodler (60). By the injection of water-soluble extracts of adrenal cortex, she obtained undescribed *modifications dans l'aspect des ovaires* of the guinea pig, while the external genitalia showed marked masculinization. The changes produced by the same treatment in ovariectomized female guinea pigs were much less marked, but a pronounced effect on the accessory sex organs of the castrated male guinea pig was observed. It seems that these results may have considerable significance in connection with adrenal virilism.

In view of the possible relationship between the adrenal cortex and the pituitary gonadotropic hormones, it is interesting to note that

a definite relationship has been established between the adrenal and the pituitary ketogenic factor. MacKay & Barnes (61) and Fry (62) have shown that the action of the ketogenic hormone is abolished or reduced as a result of adrenalectomy, even though the animals are maintained on cortin (62). The former have also shown that the ketonuria observed in pregnant rats, which is presumably due to an excessive production of ketogenic hormone from the pituitary, can be abolished by adrenalectomy. Attention must be drawn at this point to the work of Long & Lukens who have shown that the ketonuria and glucosuria of pancreatectomized cats is reduced by adrenalectomy (63); that the development of a fatty liver in the cat after pancreatectomy is prevented by hypophysectomy and adrenalectomy; and that pituitary adrenotropic extracts, containing no thyreotropic or ketogenic activity, when injected into hypophysectomized pancreatectomized cats, cause an increased excretion of glucose and nitrogen. This effect was not observed in the absence of the adrenals (64). The adrenal principle involved appears to be distinct from cortin, since administration of large doses of the latter to adrenalectomized pancreatectomized cats did not increase the glucosuria or ketonuria (65).

THE ACTION OF THE GONADAL HORMONES UPON THE ANTERIOR HYPOPHYSIS

Action upon the gonadotropic activity.—Recently the accepted theories concerning the action of the gonads on the gonadotropic activity of the anterior pituitary gland have been considerably modified. Following the work of Moore & Price (66) in 1932, the theory that the gonadal hormones suppressed the gonadotropic activity of the pituitary was widely accepted. Hohlweg (67), however, two years later, and subsequently many others found that oestrogens administered to female rodents led to the formation of corpora lutea. These findings resulted in a general acceptance of the view that oestrogens stimulated the secretion of "luteinizing hormone" by the pituitary. However, subsequent work was to prove that the gonad-pituitary relationship is more complex than was originally supposed. Lane (68) was able to show that, whereas administration of oestrin to immature rats for short periods resulted in an increase in the total number of follicles in the ovaries and an increased gonadotropic hormone content of the pituitary, prolonged oestrin treatment decreased the total number of follicles, increased the percentage of vesicular follicles, and decreased the gonadotropic hormone content of the

pituitary. This suggested that oestrin has a dual effect on the pituitary, first stimulating the production of follicle-stimulating hormone, then inhibiting its production while stimulating the production of the luteinizing principle.

During the period under review it has been demonstrated by many workers (confirming earlier work) that oestrogens, including oestrone, oestriol, oestradiol monobenzoate, emmenin, and 9,10-dihydroxy-9,10-di-*n*-propyl-9,10-dihydro-1,2,5,6-dibenzanthracene, will cause pituitary enlargement when administered to rats of both sexes (59, 69-76). On the other hand, Bachman (77) and Zondek (78) did not observe pituitary enlargement in female rabbits after oestrin administration, and the latter, while finding pituitary enlargement in male rats after oestrin, did not observe this with females. Tumours of the pituitary, as a result of prolonged treatment of rats with large doses of oestrin, have been observed by Cramer & Horning (79), Zondek (78), and McEuen, Selye & Collip (72). The histological changes in the pituitaries of rats caused by oestrin administration have been studied by Wolfe & Chadwick (70), Halpern & d'Amour (71), Desclin & Grégoire (80), Mazer, Israel & Alpers (81) and Cramer & Horning (82).

The view that oestrogens may stimulate, under certain conditions, the production of gonadotropic hormone by the pituitary has received support from Nelson (69) and Ellison & Burch (59). They have reported the production of corpora lutea in the ovaries of intact immature rats, but not in hypophysectomized immature rats, by oestrin injection. Also Mazer, Israel & Alpers (81) have reported corpora lutea formation in rats and follicular maturation in rabbits resulting from brief treatment with oestrogens. It seems probable that oestrogens may exert a direct effect on the ovary besides the indirect effect through the pituitary, since Selye & Collip (83) found that oestrone caused ovarian enlargement when administered to hypophysectomized rats whose endocrine organs were prevented from atrophy by the administration of anterior pituitary extracts. Prolonged treatment with oestrogens, on the other hand, undoubtedly results in atrophy of the gonads, presumably as a result of inhibition of the gonadotropic activity of the pituitary (71, 78, 81). Zondek (78) ascribes the ovarian atrophy resulting from prolonged oestrin treatment to a blocking of the pituitary secretion from the blood stream rather than to an exhaustion of the gland, since the pituitaries of oestrin-treated animals were found to contain normal amounts of follicle-stimulating

hormone. Inhibition by oestrogens of the production of follicle-stimulating hormone by the pituitary is also suggested by the work of Frank & Salmon (84) and of Jones & MacGregor (85) who have reported that the administration of large doses of oestrin to women past the menopause results in a disappearance of the gonadotropic principle from the urine. Halpern & d'Amour (71) have advanced the reasonable hypothesis that the pituitary enlargement caused by oestrogens is a compensatory hypertrophy following the initial stimulation and exhaustion of the gland. The low gonadotropic activity of such hypertrophied glands is due, it is suggested, to the fact that the energy of the gland is directed towards proliferation rather than to secretion. These workers have excluded the possibility that the ovarian atrophy resulting from prolonged treatment with oestrogens is the result of a direct inhibition of the action of the gonadotropic hormone.

The important work of Fevold, Hisaw & Greep (86) must be reviewed in some detail. They found that oestrin augmented the effect of follicle-stimulating hormone on the ovaries of immature rats as judged by ovarian weight and the extent of luteinization. Since this effect was not observed in immature hypophysectomized rats, it was concluded that oestrin stimulates the production of luteinizing hormone by the pituitary. On the other hand, they found that oestrin did not augment the action of pregnancy-urine gonadotropic hormone,² and, since the action of the latter can be augmented by the follicle-stimulating hormone, they concluded that oestrin does not stimulate the production of follicle-stimulating hormone by the pituitary. It was also found that after prolonged oestrin treatment the response of immature rat ovaries to both follicle-stimulating and pregnancy-urine hormones was greatly decreased. It is suggested that the latter effect is the result of exhaustion of the luteinizing and follicle-stimulating hormones in the pituitary consequent to the prolonged stimulation of the gland by oestrin. They suggest on the basis of these results that corpora lutea formation which results from prolonged administration of follicle-stimulating hormone alone is caused by the latter stimulating the ovary to secrete oestrin, which in turn stimulates the pituitary to secrete luteinizing hormone. They therefore regard the effect of oestrogenic hormones on the pituitary as an important part of the regulating mechanism of the oestrous cycle. They also point

² There is much difference of opinion concerning whether or not the action of the pregnancy-urine gonadotropic principle is augmented by oestrin. This problem has been discussed at length by Selye, Collip & Thomson (88).

out the significance in this connection of the occurrence of follicle-stimulating hormone in the urine of ovariectomized women or of those past the menopause. The inference is made that in such cases there is little or no oestrin produced to stimulate the production of luteinizing hormone. It appears to the reviewers that before these conclusions can be unreservedly accepted the status of the so-called luteinizing hormone as a specific hormone must be more firmly established. Saunders & Cole (89) have shown that the augmentation of the luteinizing effect of the gonadotropic hormone of pregnant mares' serum obtained with such materials as zinc sulphate, caseinogen, and egg albumen, is similar to that obtained with the luteinizing fraction of pituitary extracts. When it is recalled that the luteinizing pituitary fraction is inactive in the absence of follicle-stimulating hormone, and must from its method of preparation contain considerably more inert protein than the follicle-stimulating fraction, the suspicion cannot be avoided that the luteinizing action of pituitary extracts may be simply a non-specific effect of the inert material which they contain. In this connection attention must be drawn to other papers in which augmentation of the effect of various gonadotropic preparations by a variety of materials is dealt with (90, 91, 92).

Action upon the adrenotropic activity.—Enlargement of the adrenal glands of rats treated with various oestrogens has been repeatedly observed (59, 69, 72, 83, 88, 93). It appears that this hypertrophy is mainly confined to the cortex, and there is complete agreement that the effect is an indirect one through the mediation of the pituitary, since in hypophysectomized animals it is not observed (59, 69, 83, 88). It is probable that the adrenal hypertrophy is caused by a stimulation of the adrenotropic activity of the pituitary by the administered oestrogens. The possibility that an ovarian influence is also involved cannot be excluded, since Selye, Collip & Thomson (88) did not observe adrenal enlargement in male rats injected with oestrin, or in female rats similarly treated unless corpora lutea were present in the ovary. Nelson (69) did not observe adrenal enlargement in ovariectomized rats after oestrin administration.

On the other hand, Leiby (93), McEuen, Selye & Collip (72), and Ellison & Burch (59) did obtain adrenal enlargement in ovariectomized rats by oestrin injection. Probably these differences are to be ascribed to differences in the dosage, the type of oestrogen employed, and the age of the animal. Prolonged treatment of animals with massive doses of oestrin may some times inhibit the adrenotropic

activity of the pituitary since Zondek (78), while obtaining adrenal enlargement in male rats with such treatment, observed slight adrenal atrophy in females. Furthermore Cramer & Horning (79) observed adrenal degeneration in intact and gonadectomized rats of both sexes following large doses of oestrin. Burrows (94) has studied histologically the effects produced in the adrenals of intact and castrated male mice by large doses of a variety of pure oestrogens. In many animals a cortical hypertrophy with the reappearance of the so-called "X-zone" was observed.

Action upon the thyreotropic activity.—Effects of oestrogenic hormones upon the activity of the thyroid gland have been reported by many observers. As long ago as 1926, Laqueur *et al.* (95) reported a rise in metabolic rate in animals treated by oestrin. Similar results on women were reported by McClendon & Burr (96) in 1929. Leiby (93) and Anderson (97) somewhat later demonstrated a hypertrophy in the thyroids of ovariectomized rats treated with oestrin, while de Amilibia *et al.* (98) found proliferative changes in the thyroid of the oestrin-treated male rat. These results suggest that the thyreotropic activity of the pituitary may be stimulated by the oestrogenic hormones under certain conditions. Zondek (78) states that conditions of oestrin administration which will cause an inhibition of the growth and gonadotropic activities of the pituitary, will stimulate the thyreotropic activity of the gland.

On the other hand, other evidence indicates that oestrogens may depress the activity of the thyroid. Sherwood *et al.* (99, 100) found that the metabolic rate of rats was decreased by oestrin administration. They suggest (100) that an effect is exerted by oestrin on the thyroid through the mediation of the pituitary. These authors also observed that following the fall in metabolic rate there is a slight rise "which is apparently an over-compensation following an inhibition of the thyroid gland." It is suggested by them that the rise in metabolic rate observed by others as a result of oestrin administration corresponds to this secondary rise. Karp & Kostiewicz (101) showed that prolonged oestrin administration caused thyroid degeneration. Kippen & Loeb (102) reported that gonadectomy in guinea pigs of both sexes caused proliferative changes in the thyroid. They suggest that this is the result of pituitary stimulation following the removal of the influence of the gonadal hormones. Kirklin & Wilder (103) showed that oestrin administered to human acromegalics reduced the severity of the symptoms of hyperthyroidism. They believe that this is the result of the

depression of the thyreotropic influence of the pituitary. Finally, Mazer *et al.* (81) found that prolonged treatment of rabbits with oestrin caused changes in the histological appearance of the thyroid indicative of lowered activity.

Action on the diabetogenic activity.—Barnes, Regan & Nelson (104) showed that the glucosuria of pancreatectomized dogs could be greatly reduced by oestrin administration, and Nelson & Overholser (105) found that the glucosuria and hyperglycemia of rhesus monkeys rendered diabetic by the administration of anterior pituitary extracts or by pancreatectomy could be similarly reduced. In both cases the suggestion is made that the effect is due to an influence on pituitary activity. Patton & Beardwood [quoted by Mazer *et al.* (81)] claim that the diabetogenic activity of the human pituitary is depressed by oestrin administration. It seems possible that the hypertrophy of the islets of Langerhans observed in rats by Cramer & Horning (79) after large doses of oestrin may be a relevant phenomenon.

Evidence somewhat against the view that the diabetogenic activity of the pituitary is depressed by oestrin has been provided by Jones & MacGregor (85) who showed that large doses of oestrin administered to women past the menopause did not affect their sugar tolerance. Furthermore, Collens *et al.* (106) showed that the glucose tolerance of human diabetics is not improved by oestrin therapy.

In considering the possible influence of oestrogenic hormones on the so-called diabetogenic principle, it must be borne in mind that it is possible that the diabetogenic activity of the pituitary is not due to a single hormone [cf. Young (107)]. The problem of (a) the rôle played by the adrenal cortex in carbohydrate metabolism and (b) the influence of oestrogens on the adrenal cortex, which are discussed in other sections of this review, must also be considered.

Action on the growth-promoting activity.—Marked inhibition of growth, as judged by body weight and size of skeleton in rats of both sexes and in cocks, has been observed by Zondek (78, 108) to result from treatment with large doses of oestrogenic hormones. Since rats dwarfed in this manner responded in growth to anterior pituitary extracts, it was concluded that the oestrin inhibited the growth-promoting activity of the pituitary. Zondek found that to inhibit the growth-promoting activity of the pituitary, larger doses of oestrin are necessary than for inhibition of the gonadotropic activity.

In considering these results it must be remembered that Riddle *et al.* (109, 110) have suggested that the growth-stimulating effects

of pituitary extracts may be due to the combined effects of the lactogenic and thyreotropic principles rather than to the action of a specific hormone.

The effect of gonadal hormones upon the factors in the pituitary which control the activity of the mammary gland are discussed in the following section.

THE ENDOCRINE CONTROL OF THE MAMMARY GLAND

During recent years much work has been carried out on the endocrine factors concerned with the development and secretory activity of the mammary glands. Since a most comprehensive review on this subject has been published by Nelson (111), only the most recent work will be dealt with in this article.

Earlier work established the fact that in the smaller laboratory animals lactation may be suppressed by oestrogenic hormones. During the period under review the same fact has been established for the cow. Folley (112) has shown that injections of oestradiol benzoate into lactating cows causes a transient decrease in milk production with slight changes in some of the constituents. Waterman, Freud & deJongh (113) greatly reduced the milk production of dairy cows by rubbing an oily solution of oestradiol benzoate into the teats. Partial restoration of the milk production was obtained by injecting prolactin. In this connection the results of Reece & Turner (114) may be of significance. They found that oestradiol benzoate injected into male rats caused an increased prolactin content of the pituitaries; this, as they pointed out, may mean either a decreased secretion or an increased production of the hormone.

Freud & deJongh (115), using ovariectomized rats, obtained only epithelial growth in the mammary gland with oestrin administration, but with oestrin and progestin they observed a differentiation of the same epithelium. This paralleled so closely the conditions prevailing in the uterus that they speak of a systemic action of both substances. However, Selye *et al.* (116) found that oestrone plus progesterone caused little more mammary proliferation in ovariectomized rats than was given by oestrone alone. Furthermore, they found (117) that massive doses of progesterone (4 mg. per day for twelve days) caused no mammary development in rats. Gardner & Hill (182), on the other hand, were able to obtain extensive duct development in castrated and intact male mice using small doses of progestin with or without oestrin. In view of these results it might appear that there are sex

and species differences in the response of experimental animals to progesterone.

Earlier work favoured the view that the pituitary was not involved in the response of the mammary glands to oestrogens and progestin. Thus Ruinen (118), Asdell & Seidenstein (119), Freud & deJongh (120), Nelson (121), and Houssay (122) reported that hypophysectomy did not alter the response of the mammary glands to oestrogens alone, or with progestin. On the other hand, the reverse was found to be the case by Selye, Collip & Thomson (123). The results obtained during the period under review support the findings of Selye *et al.* (above). Evans, Pencharz & Simpson [unpublished; quoted by Lyons & Pencharz (124)] administered purified pituitary gonadotropic hormone to hypophysectomized rats and found that "whereas the female genital tract responded to the hormones of the stimulated ovaries, the mammary apparatus showed only a poorly-developed duct system." Lyons & Pencharz (124) injected oestrogenic hormones into hypophysectomized male guinea pigs and were able to produce only limited lobule development, while the intact controls lactated and showed mammary development comparable to that of normal lactation; the presence of the pituitary was not found necessary, however, for nipple growth. Gomez & Turner (125) produced nipple development but no growth of the lobule-alveolar system of hypophysectomized male and female guinea pigs by the injection of oestrogens. Similarly Reece, Turner & Hill (126) showed that hypophysectomy of immature male and female rats inhibited the effect of oestradiol benzoate in stimulating growth of the duct system. The ingenious experiments of Selye & Collip (83) lend strong support to the view that oestrogens affect the mammae only through the mediation of the pituitary. They injected crude alkaline extracts of sheep's pituitary into female rats. In hypophysectomized animals this extract produced little mammary growth with or without oestrone, but the same treatment of intact animals showed that the oestrone very markedly synergized the action of the pituitary extract on the mammae. Furthermore, mammary growth was absent in the hypophysectomized animals even when the ovaries were as large as, or larger, than normal. These experiments appear definitely to exclude any direct effect of the oestrogenic hormones on the mammae or an effect on them through the ovary, showing as they do the indispensability of the pituitary.

Hitherto it was believed that the male gonads or their secretions

had no effect on the mammary gland but during the period under review this idea has been definitely disproved. Selye, McEuen & Collip (127) injected testosterone benzoate into normal and gonadectomized immature male and female rats and observed slight development of acini and the presence of milk in the ducts. They hesitated, however, to exclude stimulation of the pituitary as a cause. Nelson & Gallagher (128) produced a remarkable proliferation of the mammae, consisting of complete development of the ducts and lobule formation, in ovariectomized rats by injecting androstanediol. The acini showed definite "secretory activity" but no lactation. In similar doses androstenedione produced similar changes but in a lesser degree, while androsterone was inactive. McEuen, Selye & Collip (129) showed that castration of immature male rats prevented duct development and the appearance of milk secretion and that castration of mature male rats caused a progressive involution of the gland and disappearance of milk from the ducts. Injection of testosterone in amounts sufficient to induce mammary development in ovariectomized females caused no vaginal cornification so that the testosterone could not have been first converted into an oestrogenic hormone. They conclude that such mammary stimulation as they observed is a physiological effect of the male gonad. However, in view of the effects of oestrogenic hormones on the pituitary (see previous section) and the evidence that oestrogens stimulate the mammary gland through the pituitary, there is some reason to believe, in the opinion of the reviewers, that the effects of the male gonads and of androgenic hormones might be the result of pituitary stimulation.

Disturbances in, or cessation of, lactation in animals submitted to partial or total adrenalectomy have been repeatedly observed in the past. There is, however, some difference of opinion as to whether cortin alone is adequate to support lactation in adrenalectomized animals. Swingle & Pfiffner (130) were able to support lactation in adrenalectomized dogs with cortical extracts, and Britton & Kline (131) in this year made similar observations on rats. However, Carr (132) failed to support lactation in adrenalectomized rats with doses of a Swingle-Pfiffner extract sufficient to keep the animals alive. Hartman *et al.* (133) stated that their cortical extract would not support lactation in rats and Brownell, Lockwood & Hartman (134) went so far as to postulate a special principle in the adrenal, "cortilactin," essential for lactation.

Gaunt & Tobin (135) have clarified the situation somewhat. They

found that whereas minimum life-maintaining doses of cortin were inadequate to support lactation in adrenalectomized rats, lactation was nearly normal if salt was supplied to the animals. Doubling the dose of cortical extract also served to support full lactation. Simultaneous administration of prolactin and the minimum life-maintaining dose of cortin was no more efficacious in supporting lactation than was cortin alone. These experiments seem to exclude the possibility that adrenalectomy interferes with lactation by affecting the pituitary. They also form strong evidence against the existence in the adrenal of a special lactation principle.

If prolactin were the only principle in the pituitary necessary to initiate and support lactation it should be effective in hypophysectomized animals. Previous work bearing on this question has yielded discordant results, but during the period under review the investigations have all shown that prolactin does not possess its normal action in hypophysectomized animals. Gomez & Turner (136) could elicit no response to purified prolactin preparations in the crop gland of the hypophysectomized pigeon. The same authors (137) also found that prolactin was inadequate to initiate or maintain lactation in hypophysectomized guinea pigs. However, they found that suspensions of whole sheep's pituitaries would support lactation in the hypophysectomized animal. This finding does not necessarily indicate that there is a second hypophyseal factor concerned with lactation. As the authors themselves suggest, it may only indicate the importance in lactation of the other endocrine glands which are controlled by the pituitary. The findings of Selye, Collip & Thomson (138) are not, however, so easily explained. These workers were unable to maintain lactation in the hypophysectomized rat by the daily implantation of fresh rat pituitaries. Further work is necessary before a decision can be arrived at as to the rôle of the pituitary in lactation.

It is an old observation that suckling provides a stimulus to mammary activity. During recent years much has been done towards the elucidation of the mechanism of this stimulation [cf. Nelson (111)]. Most of the investigators considered it likely that the suckling induced secretion of prolactin by nervous stimulation of the pituitary. The work of Desclin (139) is of interest in this connection. Rats were ovariectomized the day after parturition and the litters removed from half of them; at necropsy two weeks later it was found that the ones suckling young had pituitaries histologically identical with those found in pregnancy while those without litters had pituitaries typical of cas-

tration. The maintenance of the histological structure of the pituitary is attributed to nervous influences from the mammae caused by suckling. It is possible that the histological appearance of the pituitary may bear some relation to its rôle in maintaining lactation.

Most schools, notably those of Turner and Nelson, have maintained that the only direct effect of prolactin on the mammary gland is to stimulate it to secretory activity. Chaikoff & Lyons in 1935 (140), however, failed to excite mammary secretion in pancreatectomized bitches maintained on insulin. Nelson *et al.* (141) have thrown much doubt on these results by showing that lactation in pancreatectomized bitches always results from the injection of prolactin provided that the mammary gland has been sufficiently developed, i.e., if the prolactin is injected at a point in the sexual cycle when there is mammary proliferation. Thus the pancreas appears to play no special rôle.

Many specific effects of the lactogenic hormone have recently come to light. Bates, Lahr & Riddle (142) succeeded in producing broodiness in laying hens by injecting prolactin and attributed to it the rôle of causing hens to "sit." Burrows & Byerly (143) added support to this theory by showing that the pituitaries of broody hens contained more prolactin than those of laying hens or males. They also (144) found that the pituitaries of laying hens of a broody genetic constitution contained more prolactin than those of laying hens of a non-broody genetic constitution. Riddle, Lahr & Bates (145) extended their studies on the "maternalizing" influence of prolactin to mammals and found that the injection of prolactin into young virgin female rats previously "primed" with gonadotropic hormones resulted in the appearance of maternal behaviour towards young placed with them. On the other hand, Leblond & Nelson (146) observed that the hypophysectomy of mice suckling their young resulted in only a transient disappearance of maternal behaviour. They therefore regard the pituitary as being non-essential for maternal behaviour. McQueen-Williams (147) in 1935 had shown that thyroidectomy of male rats produced maternal behaviour and later in the same year (148) was able to demonstrate a decreased prolactin content in the pituitaries of such animals which he interprets as an increased secretion.

Reece & Turner (149) have studied the prolactin content of rat pituitaries under various conditions. In the male rat, age or the administration of oestrogenic hormones was found to increase the weight of the pituitary, but the prolactin content was found to remain

constant. Thyroxine, on the other hand, decreased both the weight of the gland and prolactin concentration. This is of particular interest in the light of McQueen-Williams' work referred to above. The immature female was found to possess a larger pituitary, containing a much greater concentration of prolactin, than the male of the same age. Growth was found to result in an increased pituitary weight and prolactin concentration. In the first half of pregnancy the pituitary prolactin content was found to be lowered but returned approximately to normal at term. After parturition the prolactin concentration suddenly rose to double that found at term. Thereafter there was a gradual decline in concentration.

The effect on the gonads, attributed to prolactin by several workers, is of considerable interest and importance. Riddle & Bates (150) using doves, and Bates, Lahr & Riddle (151) using hens, found that prolactin caused a rapid and marked decrease in the weight of the gonads of adult birds. Engelhart (152) observed marked changes in the ovaries of rabbits given prolactin. No ruptured follicles or corpora lutea were observed but there were many atretic and primordial follicles present, and a diffuse luteinization of the whole ovary. Dresel (153) succeeded in suspending oestrus in mature, non-parous mice for twenty to twenty-five days by injecting prolactin, but despite the continued injections a prolonged (four to eight days) oestrus followed. Selye, Harlow & McKeown (154) and Selye, Collip & Thomson (88) injected oestrin into lactating rats and found no appearance of vaginal oestrus. However, they considered the inhibition of oestrus to be due to the presence of large corpora lutea. Lahr & Riddle (155) injected prolactin into mature female rats and obtained the suspension of two to four oestrus cycles depending on the size of the dose. Large, undegenerated corpora lutea which might have inhibited oestrus were observed in the ovary, but on injecting progesterone there occurred no more than a slight prolongation of dioestrus. It would thus seem possible from the above work that the lactogenic hormone is responsible for the anoestrus of lactation in mammals. In contrast to the findings mentioned above, Votquenne (156) induced typical vaginal oestrus by the injection of oestrin during the first five days of lactation.

In considering the physiological rôle of prolactin, the recent work of Young (157) may prove to be of great interest and importance. While investigating a factor in the anterior pituitary which increases the resistance of rabbits to insulin by exerting a hyperglycemic effect

following the initial hypoglycemia, he was led to suggest as a possible explanation, that this factor renders liver glycogen more sensitive than normal to glycogenolytic stimuli such as hypoglycemia. This factor was found to be concentrated in the prolactin fraction of pituitary extracts and Young's comment on this coincidence is very suggestive:

When one recalls, however, that the period of lactation is a time when hypoglycaemia is a constant potential danger, the association of a factor stimulating lactation with that inducing an exaggerated glycaemic response to hypoglycaemia becomes of great interest.

THE MECHANISM OF "ANTI-HORMONE" FORMATION

As a result of the fundamental researches of Collip and his co-workers and of others, it is now a well-established fact that animals become refractory to certain hormone preparations, particularly those of hypophyseal origin, after their prolonged administration. Furthermore, it has been shown that the sera of such refractory animals will passively immunize another animal to the hormone in question. The early experimental findings, which have recently been comprehensively reviewed by Collip (158), led him in 1934 (159, 160) to propound his theory of anti-hormones. He pointed out that

the production of a serum inhibitory to a specific hormone may be viewed in one of two ways. Either the administered hormone extract is acting as an antigen and the inhibitory substance which can be detected in the blood serum of the treated animal is an antibody, or else the inhibiting substance represents a normal constituent of the blood which under normal conditions is balanced, as it were, against the respective hormone in such a manner as to be masked itself.

Collip preferred the latter possibility and implied that the normal hormonal state of an animal was the result of a delicate balance between the hormones and their respective anti-hormones which were produced in the body as a result of the presence of the former. If this is the case it should be possible, (a) to demonstrate the presence of small quantities of anti-hormones in the blood of normal untreated animals, and, (b) to "immunize" an animal with a hormone extract prepared from glands from its own species. The available evidence bearing on these two points is contradictory and somewhat inconclusive.

a) According to Collip (159)

The pituitary of the guinea-pig as compared with that of the rat contains relatively very little of the thyreotropic principle; the thyroid of the guinea-pig as compared with that of the rat is relatively inactive; the metabolism of the

guinea-pig is at a lower level than that of the rat; the guinea-pig is exceedingly sensitive to administered thyreotropic hormone, while the rat is extremely resistant. However, the hypophysectomized rat is exceedingly sensitive to this hormone.

This would suggest that the resistance of the rat to thyreotropic hormone is due to the high content of the anti-thyreotropic principle in the blood. However, Collip & Anderson (161) failed to demonstrate the presence of the anti-thyreotropic factor in the blood of normal rats even when hypophysectomized rats were used as test animals. They also failed to find the anti-thyreotropic principle in normal horse serum. Rowlands & Parkes (162) failed to find the anti-thyreotropic principle in the blood of normal cows, rabbits, and goats, but on the other hand they reported slight inhibition of the thyreotropic hormone by normal horse and sheep blood. The presence of the anti-thyreotropic principle in normal blood has also been reported by Eitel & Loeser (163) for the sheep, and by Herold (164) for the guinea pig. Laroche and coworkers (165, 166) have claimed that certain normal human sera contain an anti-pituitary-gonadotropic principle, and also an antibody to human urinary gonadotropic hormone (complement-fixation test).

b) As early as 1930 Siebert & Smith (167) reported that the administration of guinea-pig pituitary preparations to guinea pigs caused at first a slight rise in the basal metabolic rate and then a fall, suggesting that the animals had become refractory to the thyreotropic hormone from their own species. Selye, Collip & Thomson (168) found that the ovaries of rats receiving daily implants of 1 to $1\frac{1}{2}$ rat pituitaries for sixty-eight days were normal or subnormal in weight, which suggests that a refractory state had been established by the administration of rat gonadotropic hormone. Collip³ (169) has now shown that the serum of sheep injected for long periods with sheep gonadotropic anterior pituitary extract will passively immunize rats against injected pituitary extracts prepared from the glands of several species and against the gonadotropic hormone of pregnant mares' serum, but not against the human pregnancy-urine gonadotropic hormone. Katzman, Wade & Doisy (170) administered one to two rat pituitaries daily to female rats for seven to nine months but were unable to demonstrate the development of refractoriness to the administered gonadotropic hormone. Furthermore, the serum

³ The reviewers wish to express their appreciation of the courtesy of Professor Collip in allowing them to see this paper before publication.

of the treated rats would not passively immunize immature rats against rat gonadotropic hormone, nor would it immunize guinea pigs to the thyreotropic action of rat-pituitary implants. Twombley (171) failed to demonstrate the production of an anti-human pregnancy-urine gonadotropic principle in the sera of humans treated for as long as twelve months with pregnancy-urine preparations. In this connection it should also be recalled that Smith (172) in 1927 treated hypophysectomized rats with daily implants of fresh rat pituitaries for as long as 163 days without establishing a refractory state to the gonadotropic or growth-promoting principles in the implants. However, the somewhat subnormal weight of the thyroids and adrenals of the treated animals might be taken to mean that some formation of anti-hormones to the rat thyreotropic and adrenotropic hormones had occurred. Witschi & Levine (173), Martins (174), and Dushane, Levine, Pfeiffer & Witschi (175) found that in parabiotic rat twins, one of which was gonadectomized, an overstimulation of the ovary, with constant oestrus, occurred in the other as a result of the greatly increased output of gonadotropic hormone in the first twin following the gonadectomy. The ovaries of the intact twin remained in this overstimulated condition and the condition of constant oestrus persisted for long periods, indicating that no anti-gonadotropic hormone was produced in response to the hyperactivity of the pituitary.

Evidence against Collip's view regarding the nature of anti-hormones has been advanced by Werner (176) and by Twombley (171). The former has found that whereas he was able to induce a refractory state in male guinea pigs to a thyreotropic preparation made from beef pituitaries by the sodium sulphate method of van Dyke & Wallen-Lawrence, an extract equally potent as regards thyreotropic potency, made from beef pituitaries by the flavianic acid method of Evans, Meyer & Simpson, would not induce refractoriness. Furthermore, it was found that guinea pigs which had been made refractory to the former preparations would still respond to the flavianic acid product as judged by increased basal metabolic rate and the histological appearance of the thyroid. Werner also was able to induce refractoriness in guinea pigs to the van Dyke-Wallen-Lawrence preparation by prolonged administration of small doses, insufficient in themselves to stimulate the thyroid; no refractoriness to the flavianic acid product was obtained in a similar experiment. Finally, Werner showed that the serum of sheep and rabbits which had had prolonged treatment with the flavianic acid preparation has no inhibitory action towards

the latter when injected into guinea pigs. Werner concludes that "as regards the thyreotropic principle the alleged anti-hormone formation is an immune response to proteins associated with the hormone." Twombly, working with pregnancy-urine gonadotropic hormone, showed that preparations nearly completely inactivated by heat and completely inactivated by age, were as efficient in producing immune sera on injection as active preparations.

Parkes & Rowlands (177) have shown that the serum of rabbits made refractory to ox anterior pituitary gonadotropic preparations will passively immunize rabbits in oestrus not only to ox pituitary extracts but also to horse and sheep pituitary extracts. Furthermore, such rabbits were shown to be passively immunized against the gonadotropic hormone secreted by their own pituitaries, since ovulation did not occur after copulation. This result may be considered to constitute evidence favouring Collip's original theory, but it is by no means conclusive.

Gegerson, Clark & Kurzrok (178) have carried out experiments which they believe constitute evidence that the anti-hormones present in the blood of rabbits treated with human pregnancy-urine gonadotropic hormone or with bovine pituitary extract are distinct from the "anti-proteins" formed. The immune sera were treated with normal human male serum and bovine serum respectively, and, after centrifuging off the precipitate formed, the supernatant fluid was found still to possess anti-gonadotropic properties. The significance of these results appears doubtful to the reviewers since apparently no particular precautions were observed to attain optimum conditions for complete precipitation. Furthermore, the tacit assumption that the human and ox sera contain the same antigens as human pregnancy-urine preparations and ox pituitary extract does not seem to be entirely justifiable. These authors also found that whereas ox pituitary antiserum would not inhibit the action of human pregnancy-urine hormone, antisera to the latter would inhibit the action of bovine pituitary extract. The latter finding is hard to harmonize with the earlier findings of Selye, Collip & Thomson (179) who found that rats made refractory to pregnancy-urine hormone would still respond to pig pituitary extract.

Black (180), studying the anti-ketogenic principle, the existence of which was first demonstrated by Black, Collip & Thomson (181), showed that rats which had become immunized to the ketogenic action of ox pituitary extract failed to exhibit a marked acetonuria after

phlorhizin poisoning. This suggests that the anti-hormone to the bovine hormone also inhibits the action of the animal's own ketogenic hormone. However, these experiments do not seem to constitute conclusive evidence in favour of Collip's theory of anti-hormone action.

In the opinion of the reviewers the direct and positive evidence bearing on the question of the physiological significance of anti-hormones can be summarized as follows:

Supporting Collip's original theory:

- a) Rats develop immunity to the gonadotropic effect of rat pituitary implants (168).
- b) An immune serum may be developed in sheep by the injection of sheep pituitary gonadotropic preparations (169).

Supporting the view that anti-hormones are antigens formed in response to administration of a foreign protein:

- a) The power of beef pituitary thyreotropic extracts to induce anti-hormone formation in the guinea pig depends upon the method of preparation (176).
- b) Inactivated human pregnancy-urine gonadotropic preparations are as effective in inducing anti-hormone formation as active preparations (171).

Most of the remaining available evidence is either negative and direct or positive and indirect. It is just conceivable that the mechanisms of anti-hormone formation for various pituitary hormones differ from one another. Thus, from the evidence summarized above, it is difficult to escape the conclusion that the anti-pituitary gonadotropic principle is an anti-hormone in the original sense of Collip. On the other hand, the evidence that the anti-hormones to the thyreotropic hormone and the pregnancy-urine gonadotropic hormone are merely antigens produced in response to the injection of a foreign protein seems overwhelmingly convincing. It seems to the reviewers that a final decision concerning the nature of anti-hormones must await a better understanding of the chemical nature of the hormones involved and a fuller knowledge of the mechanism of antibody formation to natural antigens.

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DEPARTMENT OF BIOCHEMISTRY
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THE VITAMINS*

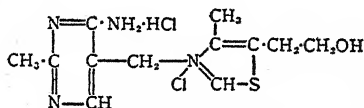
BY CAROLINE C. SHERMAN AND HENRY C. SHERMAN

Department of Chemistry, Columbia University

In accordance with growing editorial policy, we here forgo, to an even greater extent than in the preceding reviews, any attempt to mention in the brief space available all the worthy work on vitamins which has appeared within the year. Nor have we attempted the perhaps equally impossible task of assessing the relative merits of so many and such new contributions. Abandoning any attempt at either completeness or authoritative selection, we seek rather to review, in as nearly a coherent manner as circumstances permit, the contributions with which we have been able to become sufficiently acquainted.

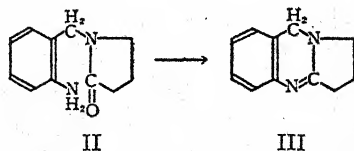
VITAMIN B₁

Chemistry.—The structural formulae for vitamin B₁ proposed in 1935 by Williams (1) and by Windaus, Tschesche & Grewe (2) have been replaced (3) by I, below. Early in the year, Makino & Imai (4),



I. Vitamin-B₁ hydrochloride

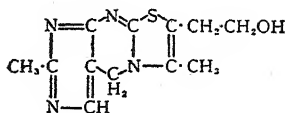
from spectrographic and other evidence, suggested that a saturated carbon atom was interposed between the thiazole and the pyrimidine nuclei. They pointed out that with this representation¹ an analogy for the formation of thiochrome from vitamin B₁ is to be found in the reaction described by Späth & Platzer (5) in which substance II is convertible into III. In a further development of this argument, Imai (6)



* Received January 18, 1937.

¹ In this paper, the methyl group was tentatively assigned to position 4 on the pyrimidine nucleus, but later (6) the 2-position was adopted in accord with other workers.

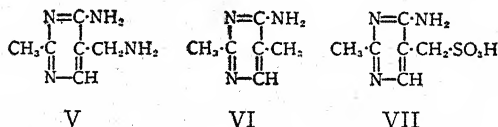
called attention to the fact that the synthetic product with the structure assigned to thiochrome on the basis of Windaus' earlier formula for vitamin B differed from natural thiochrome in showing no blue fluorescence in daylight. Bergel & Todd (7) made the same observation with regard to the product corresponding to Williams' 1935 formula for vitamin B. The latter authors (8) have since demonstrated by synthesis the structure of natural thiochrome (IV), thus affording independent confirmation of the new structural formula for vitamin B. For details of syntheses of thiochrome and other sub-



IV. Thiochrome

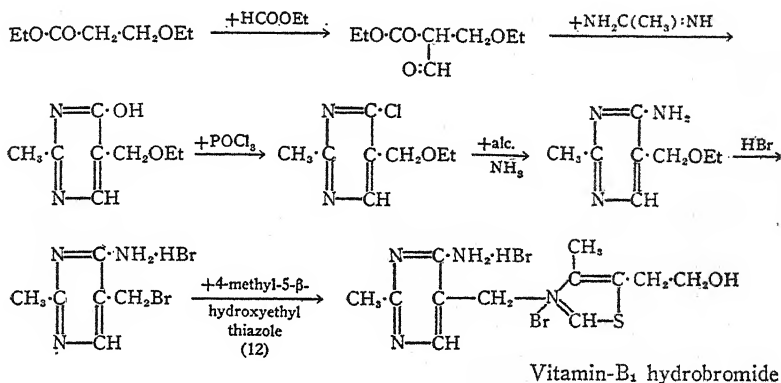
stances of interest in relation to vitamin B, see the series of papers by Todd, Bergel, *et al.* (9).

Williams (3) derived from vitamin B by treatment with liquid ammonia a base, $\text{C}_6\text{H}_{10}\text{N}_4$, which, from spectrographic considerations, was believed to be V. From the pyrimidine sulfonic acid derivative, formed by cleavage of the vitamin with sulfite, Williams obtained another base, $\text{C}_6\text{H}_9\text{N}_3$, identical with synthetic 2,5-dimethyl-6-aminopyrimidine (VI). In addition to this evidence for the existence of a CH_2 grouping between thiazole and pyrimidine in vitamin B, and for the attachment of the methyl group to position 2 of the pyrimidine nucleus, Grewe (10) simultaneously identified Williams' sulfonic acid cleavage product as 2-methyl-4-amino-5-pyrimidyl sulfonic acid (VII).



According to Grewe (10), vitamin B was synthesized early in 1936 by Andersag & Westphal, but we have not seen an account of this work in the scientific literature. In August, 1936, Williams &

Cline (11) reported their successful synthesis of vitamin-B₁ hydrobromide, through the following steps:



Determination.—Schlutz & Knott (13), and also Coward (14), have proposed a shorter test period in the rat-growth method of measuring vitamin B. Dann (15) has described a modification of the Smith rat-curative method in which the material for assay is given orally. The validity of the day-dose principle in the curative method of assay, and its application to crystalline preparations, have been critically discussed by Heyroth (16), using the rat, and by Kinnersley & Peters (17), using the pigeon. An interesting observation of the latter investigators was that the duration of cure bore no quantitative relation to the amount of vitamin given when crystalline preparations were used. Applying the catatorulin test, they found that 2 $\mu\text{g.}^2$ of vitamin-B₁ hydrochloride was equivalent to 1 International unit. This is to be compared with a value of about 2.7 $\mu\text{g.}$ as determined by the bradycardia method [Harris & Leong (18)].

Prebluda & McCollum (19) have described a highly sensitive and apparently specific chemical test for vitamin B₁, which is also adaptable to quantitative estimations: Solutions of *p*-amino-acetanilid or methyl-*p*-aminophenylketone (*p*-amino-acetophenone), which have been treated with nitrous acid, form with vitamin B under certain conditions a characteristic purple-red compound which is stable and highly insoluble in water.

Relation to carbohydrate metabolism.—Peters (20) has reviewed

² 1 $\mu\text{g.}$ = 1 γ = 0.001 mg.

both the older and more recent investigations in his department at Oxford which support the view that vitamin B₁ acts as a catalyst at some stage in the intermediary metabolism of carbohydrate in the central nervous system. Thus, brain tissue from pigeons showing acute opisthotonus symptoms takes up less oxygen in the presence of added lactate or pyruvate than does normal tissue respiring in the presence of the same substrate; and addition of vitamin B₁ *in vitro* largely restores the oxygen uptake to normal, increasing the rate of disappearance of pyruvate (but not of lactate). Peters now considers the *acute* nervous symptoms of vitamin-B₁ deficiency to be definitely related to this "biochemical lesion," i.e., failure of normal carbohydrate metabolism in some regions of the brain; and this not through any toxic effect of accumulated lactate or other metabolite, but because "the absence of an important factor in the development of energy from carbohydrate would be sufficient to stop the normal functioning of some groups of nerve-cells."

Similar effects, although much less marked and less easily demonstrated, were observed by O'Brien & Peters (21) in vitamin-B₁-deficient mammalian (rat) brain.

In respiration studies on brain and other tissues from polyneuritic chicks, Sherman & Elvehjem (22) observed anomalies of pyruvate metabolism similar to those described in vitamin-B₁-deficient pigeon brain. Lactate metabolism (23), however, was not demonstrably abnormal in chick-brain tissue, although in heart muscle diminished oxygen uptake and lactate removal were noted. Addition of pyruvate inhibited lactic acid dehydrogenase activity in both heart and kidney tissue; and it was suggested that the failure of lactate metabolism was thus a secondary effect of the avitaminosis.

Polyneuritic chicks also showed a diminished pyruvate tolerance *in vivo* as evidenced by the less rapid removal from the blood and the increased excretion of bisulfite-binding substances, following the injection of pyruvate (24).

Pyruvic acid has been isolated by Johnson (25) from the blood of vitamin-B₁-deficient pigeons, accounting for the increased bisulfite-binding capacity noted by Thompson & Johnson (26). The presence of pyruvate in the blood and cerebrospinal fluid in beri-beri has been reported by Platt & Lu (27), although no pyruvate could be demonstrated in human blood in "ordinary diseased conditions" (28).

Krebs (29), in a preliminary note, suggests the following as probable steps in the anaerobic breakdown of carbohydrate:

- I. 2 pyruvic acid + water \rightarrow acetic acid + carbon dioxide + lactic acid
- II. $\begin{matrix} \text{acetic acid} \\ + \\ \text{pyruvic acid} \end{matrix}$ + 'ketonic acid' \rightarrow α -ketoglutaric acid + 'hydroxy acid'
- III. α -ketoglutaric + 'ketonic + water \rightarrow succinic + carbon dioxide
acid' acid + 'hydroxy acid'

He states that experiments on tissues of vitamin-B₁-deficient rats and chickens indicate that vitamin B₁ is a coenzyme for dismutations of the type of reactions I and III. However, McGowan & Peters (30) report that no effect of vitamin B₁ was observed in tests of reaction III in avitaminous pigeon tissue with pyruvic acid as the 'ketonic acid.' Sherman & Elvehjem (31) could show no relation of vitamin B₁ to anaërobic glycolysis in chick cerebrum, heart, kidney, liver, and muscle.

Birch & Mapson (32) found that the administration of adenine nucleotides to normal rats produced bradycardia in all respects similar to that characteristic of B₁-avitaminosis. They suggested that the heart symptoms of this condition, and quite possibly the disturbed carbohydrate metabolism as well, are due to accumulations of adenylic acid through failure to convert it into innocuous products, as by deamination to inosinic acid, or by phosphorylation to adenosine triphosphate. It could be demonstrated directly that the former reaction, i.e., the deamination, proceeds at a subnormal rate in B₁-avitaminotic cardiac tissue. Whether the latter reaction (phosphorylation) is affected was not determined, but it is of great interest in connection with the observation (20) that pyrophosphate augments the effect of vitamin B₁ on tissue respiration, as well as the prominence of adenosine triphosphate in schemes for carbohydrate metabolism generally.

"Insulin-like" properties were ascribed to vitamin B by Lajos (33) who found that injection of the International Standard preparation, together with glucose, into fasting rats caused increases in muscle and liver glycogen similar to those induced by insulin, and some decrease in blood sugar. It will be recalled that Vorhaus, Williams & Waterman (34) noted instances of increased utilization of carbohydrate in diabetic individuals treated with large doses of crystalline vitamin B.

Clinical.—In confirmation of the work of Vorhaus, Williams & Waterman (35), successful treatment of neuritis of varying type and

probable origin with vitamin B₁, by mouth or parenterally, has been reported by Russell (36), Theobald (37), and Leak (38).

Alvarez and associates (39) detected no definite changes in the gastric juice as the result of confining human subjects for two to six weeks to a diet markedly deficient in vitamin B₁; a decrease of 11 per cent in the hemoglobin content, and 7 to 8 per cent in the erythrocyte count, was, however, reported.

Studies of urinary excretion of vitamin B.—In studies of the same general sort as those on the "saturation" of the body with vitamin C, Harris & Leong (18) find that, with vitamin B also, the level of urinary excretion on the customary diet of the individual reflects more or less closely the level of dietary intake of the vitamin; the response to a large test dose is appreciably graded, according to the past dietary history. An "average normal" value of 20 International units of vitamin B (54 μ g., according to their experience) was found for the daily excretion by adult subjects; it was suggested that excretion of less than 12 International units is presumptive evidence that the diet contains a subnormal allowance of vitamin B₁. Seven subjects with beri-beri excreted less than 2.5 International units daily, despite the fact that treatment with diets of high vitamin-B₁ content had already been initiated. In contrast with vitamin C, the vitamin B eliminated in the urine even with high intakes corresponds to only 5 to 8 per cent of that in the food. In these studies, acid clay was used to adsorb the vitamin B from the urine, and the subsequent assay was made by the bradycardia technique. Roscoe (40), who estimated the vitamin-B content of the urine by its effectiveness in curing neuritis in rats, concluded that the variations in the excretion by normal individuals on the same basal diet in response to test doses seemed sufficiently great to discourage the application of the "balance-sheet" method to the study of vitamin B.

Knott (41) has reported a quantitative study of the utilization and "retention" (retention plus catabolism?) of vitamin B by eight children of four to seven years of age with different levels of intake, and concludes that, for these children, maximal "retention" (difference between the amount in the food and the amount in the excreta) required the feeding of about 200 times as much vitamin B as is needed to support one gram per day of gain in the standardized young rat. Whether maximal retention in this sense and maximal weight-gains thus induced are optimal in their effects upon the entire life cycle, apparently remains to be investigated. Such investigation seems

to be particularly needed in the case of vitamin B₁; for the urging of extra high food consumption by the extra liberal feeding of this specific appetite stimulant is clearly put upon the defensive by the point of view which questions the "forcing" of growth. Each nutrient which is found to accelerate growth should be investigated separately, on its own merits, as to the after-effects of such acceleration.

VITAMIN B₄

In contrast to the recent rather widespread tendency (42, 43) to regard the manifestations of so-called "vitamin-B₄ deficiency" in the rat as merely a chronic B₁ hypovitaminosis which may be cured by sufficient quantities of vitamin B₁, the Wisconsin investigators have advanced evidence for the existence of vitamin B₄ as a separate entity, required for normal mammalian nutrition. By employing a ration (44, 45) more highly purified than the usual "vitamin B-free" diets, they were able to produce the typical symptoms described by Reader without first inducing the syndrome of vitamin-B₁ deficiency. They showed that, under these conditions, the symptoms failed to respond to crystalline vitamin B₁ (5 µg. daily) but disappeared when "vitamin B₄" supplements were given (45). By means of an assay on chicks (which are said to be much more susceptible to vitamin-B₄ deficiency than are rats), dried grass, peanuts, wheat germ, pork brain, and pork kidney were found to be "good" sources, and grains, "relatively poor" sources, of this factor (46). The failure of most investigators to produce B₄ avitaminosis without drastic deficiency of B₁, as well as the alleged cures of B₄ symptoms by crystalline B₁, may be due to the presence of small amounts of B₄ in the basal diets which suffice to meet the rat's requirements so long as normal food consumption continues but are inadequate for protection against vitamin-B₄ deficiency when, as the result of B₁ avitaminosis, the food intake is lowered (47).

VITAMIN B₃

Carter & O'Brien (48), defining vitamin B₃ as "a factor required for full weight restoration in the pigeon on a diet which, in addition to vitamins B₁ and B₅, includes an adequate supply of basal dietary constituents such as salt and protein," report that at least two factors appear to be involved in "vitamin B₃" as so defined: one which is

adsorbed from liver concentrates on fuller's earth, carrying with it flavin; the other, present in the filtrate after treatment with this reagent.

LACTOFLAVIN AND OTHER HEAT-STABLE FACTORS OF THE VITAMIN-B COMPLEX

Karrer (49) reports isolation of flavins from eggs, liver, kidney, malt, grass, dandelion flowers, and fish eyes—all chemically identical with lactoflavin. Both his laboratory and that of Kuhn have continued their studies on synthetic flavins, flavin derivatives, and the biological activity of these preparations (50 to 58).

Supplee *et al.* (59) have described a fluorometric method for the estimation of lactoflavin, accurate to 0.1 $\mu\text{g.}$ and capable of use for the qualitative detection of as little as 0.05 $\mu\text{g.}$ per cc. of solution.

Emmerie (60) has described a colorimetric method for the determination of flavins in urine, and has reported preliminary observations on the urinary flavin excretion of normal individuals and its variation with the dietary intake (61).

The fact developed by the earlier work of Booher and reported also by Bisbey & Sherman (62), that the flavin factor is the same which is measured as "vitamin G" by the Bourquin method, is now found by Day & Darby (63) to be true also of vitamin-G measurements made by Day's modification of this method. The latter employs an 80 per cent alcoholic extract of rice polishings while the former uses a similar extract of ground whole wheat as source of vitamins B₁ and B₆ (or H). Moreover, Day, Darby & Langston (64) have shown by experiments with both natural and synthetic lactoflavin that it is this flavin factor which prevents the rat cataract previously studied as a symptom of vitamin-G deficiency by Day and coworkers, and which Day (65) now finds to be clearly distinguishable with the ophthalmoscope from the cataract produced by the feeding of excessive amounts of lactose or galactose. Further evidence that the flavin factor is the substance which prevents the cataract of vitamin-G deficiency in rats is afforded by the correlation of growth and cataract-prevention in an extended series of quantitative experiments by Day & Darby (66).

The much higher vitamin-G (flavin factor) values found by Day & Darby (63) in the rennet-curdled American and Swiss cheeses than in the presumably lactic-acid-curdled "cream cheese" (creamed cottage cheese), while partly attributable to the difference in water con-

tent, suggests the possibility of an adsorption of lactoflavin upon casein occurring in greater or less degree with variations in the conditions of coagulation. This may bear upon the problem discussed by Supplee and coworkers (67) of the possible presence of lactoflavin in some experimental diets which hitherto were supposed to be vitamin-free.

Adams (68) reports the production, by feeding the Bourquin "vitamin-G-deficient" diet (69), of a skin condition in rats in which the rate of respiration of the skin cells was reduced; the oxygen uptake was not restored by treatment of the skin with lactoflavin prepared as described by Booher in 1934 (70). Here, as in some of the work of the Kuhn laboratory (71), the use of what is called the Bourquin diet seems to have induced a multiple deficiency, instead of simply a deficiency of the flavin factor as in the work of Day and of several workers in the Columbia laboratories. Such a difference might be due to one or more of three causes: (a) extraction with a different amount or concentration of alcohol so that less of the second limiting factor (vitamin B₆ or H?) is supplied along with the vitamin B₁; (b) a more severe depletion of the experimental animals with the result that an avitaminosis of the second limiting factor is superimposed upon the flavin deficiency; (c) differences in the nutritional background and resulting bodily stores of the experimental animals. Ansbacher, Supplee & Bender (72), working with a different basal diet and a specially prepared rice-polishings concentrate, find that the latter supplies (like the 80 per cent alcoholic extract of ground whole wheat used by Bourquin, and the rice-bran extract of Day) "a third factor or group of factors" along with vitamin B₁, so that, in the animal thus fed, growth is determined by lactoflavin and the rate of growth may be made a means of measuring the (nutritional) flavin value of a food or a concentrate.

Ringrose & Norris (73) reported in December the differentiation of an (alcohol-water) insoluble and of a soluble factor, both potent in preventing "chick pellagra." Interpretative discussion of these observations and of those of Lepkovsky & Jukes (74) which indicate a difference in response between chicks and young turkeys, is probably best deferred.

Booher & Hansmann (75) describe experiments with a whey concentrate which cured experimental blacktongue in dogs and improved the growth of rats which, without it, grew subnormally on the diet used to induce blacktongue. Failure to cure blacktongue by

lactoflavin alone was reported by Birch *et al.* (76) and by Koehn & Elvehjem (77).

The relative abundance of vitamin B₆ in cereals, including maize, reported in 1935 by Birch, György & Harris (76), has been confirmed by Dann (78) and by Copping (79).

According to Birch, György & Harris (76), vitamin B₆ is distinct from the human antipellagric and the canine anti-blacktongue factor, since the usual Goldberger blacktongue-producing diet is highly effective in curing the "specific" dermatitis of rats, while canine blacktongue and human pellagra develop in the presence of liberal amounts of vitamin B₆ (in the form of maize meal) yet are readily cured by liver extract containing (by rat assay) much less B₆. Studies using diets consisting more largely of purified materials indicated that, in addition to vitamin B₁ and lactoflavin, both vitamin B₆ and the anti-blacktongue factor are needed for normal nutrition in the dog; the suggestion was made that maize is important in blacktongue-producing diets, not, as some have supposed, because it supplies a toxic agent, but as a source of vitamin B₆, in the absence of which the dog may develop B₆ deficiency instead of blacktongue. These investigators also hold that the "chick pellagra" studied by Elvehjem & Koehn (80) is not a B₆ deficiency, this factor being contributed by the maize meal making up half the "chick pellagra"-producing diet.

Birch & György (81) noted that the greater part of the vitamin B₆ in plant and animal tissues is not easily extracted by ordinary solvents but more is liberated in a soluble state by autolysis or after digestion of the tissue with papain, which led them to suggest that "possibly the vitamin is attached to the protein as a prosthetic group which is not easily split off." They summarize their observations on the behavior of vitamin B₆ toward various chemical reagents:

(1) The vitamin is not precipitated by salts of lead, mercury or silver, or by picric acid; (2) it is adsorbed on fuller's earth from acid solution, is precipitated by phosphotungstic acid and migrates towards the cathode on electro-dialysis; (3) it is inactivated by benzylation but not by the action of nitrous acid; (4) it is soluble in ethyl alcohol but is not extracted from a concentrated watery solution by acetone, amyl alcohol or ether.

They conclude, "It is suggested that the vitamin does not contain a primary amino group but is of a basic nature and possibly contains a hydroxyl group."

In confirmation of earlier reports by György, Copping (82) observed that young rats maintained on diets lacking in flavin factor

but containing vitamin B₆ developed the type of skin lesions designated by Chick, Copping & Edgar (83) as the *b* type and by György (84) as "non-specific" dermatitis; on B₆-deficient diets containing flavin the *a* or "florid" or "specific" type of dermatitis appeared. In the former case, cures were observed in about half the animals when pure flavin was given; in the latter case, vitamin B₆, supplied as an alcoholic extract of whole maize or wheat, effected rapid and complete cures, but less regular results were obtained with B₆ preparations from yeast. Dann (78) found that exposure of rats on a vitamin-B₆-deficient ration to strong sunlight failed to increase the incidence or severity of the dermatitis as compared with rats on the same diet in darkened rooms.

The observation of Hogan & Richardson (85) of a dietary dermatitis of rats which is apparently identical with B₆ deficiency but which can be cured by wheat-germ oil may be explained by the finding of Birch & György (81) that fats (linseed oil and lard) exert a sparing action on vitamin B₆. The latter authors point out as suggestive the similarity of certain B₆-deficiency symptoms to those described by Burr & Burr (86) as resulting from lack of "indispensable unsaturated fatty acids," together with the high linoleic acid content of the fats which were effective in "sparing" B₆. However, Hogan & Richardson reported that cod-liver oil, corn oil, flaxseed oil, and walnut oil were relatively ineffective in healing the dermatitis which they studied.

Booher (87) reports that a deficiency of "vitamin H" in the diet of young rats causes cessation of growth, general debility, marked loss of hair, and a severe erythroedemic dermatosis which affects especially the feet, ears, and tail. Her experiments show that milk, whey, liver, yeast, rice polishings, and cereals generally are among the materials carrying significant amounts of this vitamin.

Koehn & Elvehjem (77) found that canine blacktongue was cured by flavin-free extracts from liver which were also effective in curing "chick pellagra." They state:

Whether the fraction which has been found active for both chicks and dogs contains only one specific active substance cannot be answered at this time. Our work on the purification of the antipellagric factor indicates that there is only one substance concerned with this deficiency disease.

The designation "filtrate factor" has been applied by Lepkovsky & Jukes to the substance active in preventing "chick dermatitis," which they have further characterized (88) and separated from vitamin B₆.

by adsorption techniques (89). In a study of the relation of both of these factors to the nutrition of the rat, they report (89) that animals receiving a basal B-deficient ration supplemented with a concentrate of vitamin B₁ and crystalline lactoflavin usually died without developing dermatitis. Those given, in addition, concentrates of the "filtrate factor" (free from vitamin B₆) grew slowly and developed an acute dermatitis; if B₆ was then supplied, growth increased greatly and the dermatitis was cured. Rats given vitamin B₆ but no "filtrate factor" showed failure of growth, "the eyelids became swollen and tended to stick together, the eyes were watery and the nose inflamed"; on addition of the "filtrate factor," the normal condition was gradually restored. This would indicate that normal nutrition in the rat requires, in addition to lactoflavin and vitamin B₆ or H, another water-soluble factor which occurs in concentrates of the filtrate factor which prevents dermatitis in chicks; they think that the two factors may be identical.

Using a method of assay based on the growth response of chicks, Jukes & Lepkovsky (90) have investigated the distribution of the filtrate factor in foods and feeding-stuffs. Their report that wheat germ and fresh kale seem to be of about the same effectiveness as corn as sources of the filtrate factor is of interest in relation to the high pellagra-preventive value ascribed to wheat germ and kale by Sebrell (91). Fouts, Lepkovsky, Helmer & Jukes (92) report that two pellagrins maintained on the Spies diet showed striking improvement when treated with a concentrate from liver which contained the chick anti-dermatitis factor but which was free from lactoflavin and vitamin B₆. Other pellagrins under the same conditions failed to respond to lactoflavin alone [as Spies & Chinn (see 76) and Ruffin & Smith (see 78) had also observed], but were cured by supplements of liver extract.

VITAMIN C

The recent literature on vitamin C through 1935 has been summarized in the excellent review of King (93).

Determination.—Coward & Kassner (94) discuss a method for bioassay of vitamin C in which the growth of guinea pigs during a six weeks period is compared with that resulting from administration of the International Standard at different levels; they conclude that the "tooth" method is preferable, being fully as accurate as the growth method, more specific, and less time-consuming.

The indophenol-titration method has been reinvestigated both with respect to the possible interference of non-vitamin reducing materials and in relation to reversibly oxidized vitamin C, which is not estimated by direct titration. The suggestion has been made (95) that by titrating at 0°C. the oxidation is slowed sufficiently so that excessive haste becomes unnecessary and more constant endpoints may be obtained. Van Eekelen & Emmerie (96) restate their belief that preliminary precipitation with mercuric acetate is necessary to the specificity of the titration technique, the danger that thiosulfate may otherwise introduce serious error being stressed by them and by Heinemann (97, 98). They also consider that preliminary reduction with hydrogen sulfide, which is a necessary step in their procedure, is always desirable in view of the ease with which vitamin C may undergo reversible oxidation in the course of preparing tissue extracts. The problem of the ascorbic-acid-oxidase activity of many vegetable tissues has been studied by Kertesz, Dearborn & Mack (99), from the viewpoint of preservation of vitamin-C values in stored foods, and by Musulin & King (100), in relation to the determination of vitamin C by titration methods. The latter find that the use of metaphosphoric acid with acetic or trichloroacetic acid in the extraction and titration procedures is very valuable in inhibiting this oxidase action. The catalytic effect of extremely low concentrations of copper (46 µg. per liter) on the oxidation of ascorbic acid by atmospheric oxygen, as studied by Barron, Barron, DeMeio & Klemperer (101, 102), may be of great importance in connection with the loss of nutritional value in the handling of foods and also in the determination of vitamin C by current titration methods. In fluids of animal origin and some vegetable fluids, vitamin C is protected against copper catalysis by the action of glutathione, proteins, and amino acids; in many vegetable materials such protective mechanisms are lacking (102).

Roe (103) devised a method for the determination of vitamin C based on the observation that the reduced form gives furfural on boiling with hydrochloric acid, whereas the reversibly oxidized form does not: The furfural is determined colorimetrically with aniline acetate, the difference between the yield from extracts in which the vitamin C has been reversibly oxidized (by norite) and from the same extracts after regeneration of the reduced form (by stannous chloride) being a measure of the total (reduced plus reversibly oxidized) vitamin C initially present in the extract. Oxalic acid was rec-

ommended as a preservative of vitamin C against the action of plant-tissue oxidases; in extracts so prepared close agreement was obtained between the (total) vitamin-C content determined by Roe's method and the (reduced) vitamin-C content as determined by the indophenol titration. The agreement between the two methods as applied to animal tissues was also good except in the case of liver, for which the values by the indophenol titration were 25 per cent higher than by the colorimetric technique.

The addition of excess methylene blue and back-titration with titanium trichloride was used by Gál (104) for the estimation of vitamin C. Medes (105) discusses further the colorimetric determination of vitamin C in urine by means of phospho-18-tungstic acid which she finds is of equal or greater accuracy than titration methods. The determination of vitamin C in blood is considered by Farmer & Abt (106), Taylor, Chase & Faulkner (107), and Pijoan, Townsend & Wilson (108).

Distribution and synthesis in nature.—In a study of the effect of various dietary treatments on the synthesis and storage of vitamin C in the rat, as indicated by the indophenol-reducing capacity of various tissues, Hopkins & Slater (109) reported that, although on a normal mixed dietary the concentration in liver and small intestine is about the same, when carbohydrate forms the sole organic constituent of the diet, the hepatic concentration rises and becomes higher than in the intestine. During fasting, or on a diet free from carbohydrate, the vitamin-C concentration in the liver falls, while that in the intestine increases. The explanation toward which the authors incline is that both liver and intestine are sites of formation rather than storage of vitamin C; also, that "in the rat the cells of the intestinal epithelium—unlike the hepatic cells, or much more readily than they—can, when deprived of carbohydrate, turn to protein or fat as a primary source of precursors for the synthesis of ascorbic acid." Zilva (110), repeating these experiments, found an increase in concentration but not in total content of vitamin C in the intestine on fasting, and no consistent rise in concentration on feeding carbohydrate-free diets. He concludes: "it would appear that Hopkins' hypothesis, attractive as it may be, is so far not fully supported by experimental evidence." Studies by Svírbely (111) of the vitamin-C content of rat tissues as affected by diet and various toxic substances were interpreted as indicative of "the important rôle played by the small intestine in yielding primary precursors of vitamin C." It was

also concluded that "adequate amounts of vitamin B factors are essential to obtain normal values for the concentration of vitamin C in the organs of the rat."

Guha & Ghosh (112) explain the discrepancy between their earlier findings (113) and those of Euler, Gartz & Malmberg (114) with respect to the ability of rat tissues to form vitamin C from mannose *in vitro*, by their observation that the vitamin-C content of tissues incubated with mannose under nitrogen is no greater than that of tissues incubated without mannose; while after incubation in the presence of air those with mannose showed 10 to 35 per cent more vitamin C than those without mannose, the values for both being, however, distinctly lower than in fresh tissue.

Rohmer, Bezssonoff & Stoerr (115) found a higher concentration of vitamin C in the cerebrospinal fluid of premature than of normal infants. They regard the fetus as synthesizing vitamin C, a point of view with which Mouriquand *et al.* (116) are not in agreement. Further study of this question was undertaken by Giroud *et al.* (117) with somewhat inconclusive results. Laporta & Rinaldi (118) report that according to the evidence of controlled experiments with guinea pigs neither the corpus luteum nor the fetus can synthesize vitamin C.

Giroud and associates have reviewed the "normal" values for the vitamin-C content of organs of species of animals not subject to vitamin-C deficiency (119); they regard such values as the optimum level for species which are susceptible to vitamin-C deficiency. The effects of different levels of intake (120 to 122) and of restriction to a scorbutic diet (123) on the concentration and distribution of vitamin C in the organs of susceptible species were also studied. The quantity of vitamin C necessary to maintain the normal reducing value of the liver and adrenals of the guinea pig has also been investigated by DeCaro (124).

The microtechnique, by means of which the vitamin-C content of thin tissue slices and even in some instances of single cells may be estimated, has been extended by Glick & Biskind to studies of the concentration and distribution of vitamin C in the corpus luteum (125), the adrenal gland (126), and the thymus gland (127), at various stages of development and activity; and to its occurrence in different regions of the intestine (128).

In confirmation of earlier reports (129, 130), Selleg & King (131) find the vitamin-C content of human milk to be dependent upon the intake, and, under favorable dietary conditions, to vary from 5.5 to 8

mg. per 100 cc., thus exceeding by several times the normal value for cow's milk. Riddell, Whitnah, Hughes & Lienhardt (132), from experiments in which cows on pasture were compared with those receiving dry feed alone or supplemented with silage, concluded that diet had no significant effect on the vitamin-C content of the milk, which they found to average about 2.6 mg. per 100 cc. On the other hand, in a study by Rasmussen, Guerrant, Shaw, Welch & Bechdel (133) on the effect of breed and stage of lactation on the vitamin-C value of cow's milk, distinctly lower values were reported, ranging for the different breeds from 10.0 mg. (Holstein) to 14.8 mg. (Brown Swiss) per quart; evidence was cited for the view that diet also has an effect.

The extended investigations of Tressler, King, and associates into the influence of variety, soil, and maturity on the vitamin-C values of spinach (134), peas (135), and other vegetables (136), and the effects of storage and cooking under different conditions (136, 137), do not permit of adequate summary in the space here available. Likewise mention only can be made of studies of related interest by Olliver (138), Pett (139), and Hanning (140).

The question of the increase in apparent vitamin-C value of some foods on boiling has been reinvestigated by Guha & Pál (141) and Levy (142) in experiments purported to confirm the hypothesis of a combined (non-reducing) form of vitamin C which is hydrolyzable by heat. Mack (143) concludes that the effect is mainly, if not entirely, due to the inactivation of tissue oxidases by the heating process, stating that the total value can be obtained in unheated material if acid sufficiently strong to inhibit the enzymes is used.

The "saturation" hypothesis and the relation of level of intake to health and disease.—The method suggested by Harris & Ray for estimating the state of vitamin-C nutrition of an individual from the level of urinary excretion of vitamin C while on his customary diet and from the response to large test doses of the vitamin has been widely applied, and the "saturation" hypothesis substantially confirmed (144 to 148). Harris *et al.* (149) were impressed by the large proportion of patients admitted to hospitals in Cambridge and in Manchester who showed evidence of vitamin-C subnutrition by this criterion. Similarly, Orr (150) estimated from dietary studies that about half the English population receive less than their reputed optimum allowance of vitamin C.

In Holland, on the other hand, van Eekelen & Wolff (151), in a

dietary study covering 33 families of varying economic status, estimated an average daily intake, per 70 kg. of body weight, of 91 mg. of ascorbic acid, the lowest value being 55 mg., the highest, 124 mg. This compares favorably with the estimate of 60 mg. daily to maintain a state of "saturation" (see below). Hou (152) has reviewed the problem of vitamin C in nutrition in China.

The large amounts of vitamin C necessary to fully "saturate" the body's tissues when these have been seriously depleted as in scurvy are illustrated by three scorbutic patients of Schultzer (147) who required 7.0, 9.5, and 14.4 grams, respectively.

In a series of studies (97, 153, 154), the results of which are summarized by van Eekelen, Heinemann & van Wersch (155), individuals who had been "saturated" with ascorbic acid were maintained for several weeks on a diet of known vitamin-C content, after which they were again brought to a state of "saturation." The total intake of vitamin C for such a period starting and ending with the subject "saturated," divided by the number of days, was considered to represent the daily requirement. For the four cases reported the vitamin-C requirement was 0.83 to 0.84 mg. per kilogram of corrected body weight, which corresponds to about 60 mg. for an adult of average size. From this it appears that, in order to maintain maximum tissue stores of vitamin C, the intake must be two to three times that previously estimated to "prevent the slightest objectively ascertainable prescorbutic alterations" (i.e., in capillary fragility).³

The reliability of the capillary-fragility tests as a measure of the adequacy of the vitamin-C intake is questioned further by Anderson *et al.* (156), Abt *et al.* (157), and O'Hara & Hauck (148), who report that the capillary fragility varies widely among normal individuals and is not always systematically affected by variations in the vitamin-C intake. Weld (158) noted that in many instances vitamin D was a more effective agent in increasing capillary resistance than was vitamin C.

Everson & Daniels (159) determined the vitamin-C "retentions" (i.e., intake minus urinary excretion) of three preschool boys at levels of intake ranging from 2.7 to 12.7 mg. per kg. The retentions increased with increasing liberality of intake up to about 7.5 mg. per kg., the youngest child showing the highest level of retention.

An increase in the indophenol-reducing capacity of the urine has been reported by Ahmad (160) on a diet of high purine content

³ Cf. *Ann. Rev. Biochem.*, 4, 348 (1935).

(meat, fish, extractives) but not on a high protein, low purine diet (eggs); and by Chakraborty & Roy (161) on a high fat (butter) diet and on diets rich in casein or meat. However, Heinemann (97, 98) asserts that the effect of high meat diets is due mainly to an increased elimination of thiosulfate (which reduces the indicator), the vitamin-C content [by the method of Emmerie & van Eekelen (162)] remaining essentially unaltered. He, as well as Chopra & Roy (163), failed to confirm the alleged effect of high fat diets. Hawley *et al.* (164) observed that, with both a high and a low intake of vitamin C, the urinary excretion of indophenol-reducing substances was definitely greater when the urinary acidity had been increased by the administration of ammonium chloride than when an alkaline urine was being formed as the result of ingestion of sodium bicarbonate. Therapeutic doses of acetylsalicylic acid were found by Daniels & Everson (165) to increase by more than 100 per cent the vitamin-C excretion of children.

The vitamin-C value of the blood (or plasma) has been shown to bear a close relationship to the level of intake (107, 157, 166) and to the urinary excretion (154, 166, 167) of vitamin C. In cases of scurvy, the value was usually below 0.4 mg. per cent (107); at "saturation," from 0.9 to 1.5 mg. per cent (154, 167). Stephens & Hawley (168) found that, while the concentration of vitamin C in plasma and erythrocytes was about equal, that in the white cells was many times as great. For this reason blood from patients with leukemia had an ascorbic acid content distinctly above normal (1.7 to 5.5 mg. per cent).

Several recent studies seem to indicate that during tuberculosis the requirement for vitamin C is increased. Heise & Martin (169) found that when tuberculosis patients were classified on the basis of their urinary excretion of vitamin C, there was an inverse relationship between the level of excretion and the incidence of active tuberculosis; the response to increased intakes of vitamin C showed a similar gradation with the severity of the infectious process. Hasselbach (170) reports similar findings and stresses the therapeutic importance of "saturating" tuberculosis patients with vitamin C. Comparable observations were made by Abbasy, Hill & Harris (171) in surgical tuberculosis. Deggeller (167) found that more vitamin C was required to "saturate" tuberculosis patients (who had been receiving fairly liberal allowances previously) than to "saturate" normal individuals who habitually received a diet very low in vitamin C.

According to the findings of Greene, Steiner & Kramer (172), chronic vitamin-C deficiency in guinea pigs increases the severity of tuberculosis induced experimentally under controlled conditions. On the other hand, in the experience of Heise & Martin (173), the progress of experimental tuberculosis in the course of five months was not altered significantly by "supervitaminosis-C," resulting from daily injection of 20 mg. of vitamin C into guinea pigs, as compared with control animals on a good natural diet.

A large group of juvenile patients with active or convalescent rheumatism observed by Abbasy, Hill & Harris (171) showed distinctly subnormal urinary excretion of vitamin C as compared with control subjects on the same institutional diet, suggesting that in some way the metabolic use of vitamin C is increased by this condition. The same conclusion was reached by Rinehart and associates (174, 175) who found distinctly subnormal plasma-ascorbic-acid values in rheumatoid arthritis and rheumatic fever, unless exceptionally high intakes of vitamin C had been habitual. Sendroy & Schultz (176) reported that a deficiency of vitamin C might arise as an effect of rheumatic fever through digestive disturbances; but Schultz (177) found that administration of large doses of vitamin C did not alter the incidence of the disease in children, and questioned again the thesis of Rinehart (178, 179) that vitamin-C deficiency may be an important factor in the etiology of rheumatic fever.

Bellows (180) finds that the blood and urine of people having cataract contain less vitamin C than those of normal people; more vitamin is needed by subjects with cataract, than by normals, in order to bring about an increase in the vitamin-C content of the blood plasma. This observation and much of the present-day general picture suggests that liberality of intake of vitamin C may be an important factor (though not the only one) in what McCollum & Simmonds have engagingly called "the preservation of the characteristics of youth," or in helping the body to resist the aging process.

Reversibly oxidized vitamin C.—A substance which reacts with the indophenol indicator only after preliminary reduction with hydrogen sulfide, and which is presumed to be "reversibly oxidized vitamin C" (dehydroascorbic acid), was reported by McHenry & Graham (181) to be present in certain vegetables (e.g., lettuce, string beans) in greater amounts than the reduced form. Tomatoes, lemon juice, peppers, and adrenal glands, on the other hand, were said to contain all of the vitamin C in the reduced form. According to Lee & Read

(182), the reversibly oxidized form predominates also in sprouting soya seeds. Whether this reversibly oxidized vitamin C is regarded as present in the living plant or as arising in the course of grinding and extracting the plant tissue, the fact that reversible oxidation may take place to a greater or less extent in the storing and handling of some foods before consumption gives practical interest to the question of the nutritional function of reversibly oxidized vitamin C. That reversibly oxidized vitamin C has some antiscorbutic activity was demonstrated by Hirst & Zilva (183) and has been confirmed by Fox & Levy (184) and by Roe & Barnum (185). On the basis of a quantitative comparison of the effectiveness of the two forms of vitamin C in promoting growth of guinea pigs on a scorbutic diet, Roe & Barnum (185) concluded that reversibly oxidized vitamin C is about one-fourth as active as the reduced form. It is apparently reduced in the body, Johnson & Zilva (186) having reported that individuals saturated with vitamin C excrete ingested dehydroascorbic acid in the reduced form. Roe & Barnum (185) demonstrated that the change may be brought about *in vitro* by incubation with plasma or erythrocytes, and Borsook & Jeffreys (187), by treatment with glutathione. Practically no tissue stores of vitamin C in either the oxidized or the reduced form were found by Fox & Levy (184) and Roe & Barnum (185) after administration of reversibly oxidized vitamin C. However, Bülow (188) reported equal storage, as the reduced form, whether the oxidized or the reduced form was given. According to Kellie & Zilva (189), the relatively large increase in the indophenol-reducing capacity of blood-plasma filtrates observed (190) after treatment with hydrogen sulfide is not due to the presence of ascorbic acid.

FLAVONE FACTOR ("CITRIN," VITAMIN P)

The observation was made by Szent-Györgyi and his associates (191, 192) that certain pathological conditions characterized by an increased permeability or fragility of the capillary wall, which did not respond to treatment with ascorbic acid, could be cured by fractions consisting of "practically pure flavone or flavonol glucoside." The vitamin nature of this group of vegetable dyes, the flavones or flavonols, was further indicated by studies (193) in which guinea pigs on usual "scurvy-producing" experimental diets showed a considerably lengthened survival period, and lessened decline in weight, when given 1 mg. daily of "citrin," the crystalline "flavone fraction"

of lemon juice. They died, however, after presenting the typical symptoms of experimental scurvy, and on necropsy showed fragility of bones, looseness of teeth, and swelling of joints. The severity of the hemorrhagic manifestations was, however, notably less in the guinea pigs receiving "citrin" than in control animals on the scorbutic diet alone. The authors conclude: "These results suggest that experimental scurvy, as commonly known, is a deficiency disease caused by the combined lack of vitamin C and P. . . . The vitamin P seems . . . to have a marked and somewhat specific influence on the capillary system. This agrees with our clinical observations."

VITAMIN A AND ITS PRECURSORS

As was already apparent in the review of the previous year, the establishment of the constitution of vitamin A and of its chemical relationships to its precursors appears to be regarded as essentially a closed chapter, and investigations tend either toward more distinctly physiological and pathological problems, or toward quantitative work on vitamin-A values. With respect to the latter aspect, the international investigation of standards and agreement upon a unit was fully reviewed last year. The more quantitative and definitive investigation of distribution of vitamin-A values in nature now logically follows. During the year, data on milk and butter published by Coward and Morgan have been brought together with other published and unpublished data with the result that fifty-three samples of milk show a mean of 292 ± 12 and eighty-six samples of butter a mean of 5060 ± 180 International units of vitamin-A value per 100 gm. As the probable errors are only about 4 per cent of the values of the means which they qualify, these means may be considered as fairly well established. Although butter usually contains more than twenty times as much fat as does milk, it here shows less than eighteen times the vitamin-A value. This, and the further fact that the coefficient of variation is 50 for butter as compared with 42 for milk, indicate that recovery of the vitamin-A values of milk, in butter, fails of quantitative completeness by a variable margin averaging about 10 to 15 per cent. This difference is presumably due in part to the presence of a small proportion of the vitamin-A value in the aqueous phase of the milk, and in part to a slight diminution of the vitamin value in the making and handling of the butter. Restriction of space does not permit a similar consideration of the more tentative statistical data for other foods summarized elsewhere (194).

Causes of variation of the vitamin-A values of foods are also being studied. Virtanen (195) finds the proportion of carotene in a given plant organ (and therefore its vitamin-A value) is higher the better the plant grows.

Kraybill & Shrewsbury (196) find carotene either less stable or less available when dissolved in butterfat which has been treated with Lloyd's reagent than when dissolved in ordinary cottonseed oil. Chevallier & Dubouloz (197) report the beginnings of a study of the photochemical destruction of vitamin A, review of which may await the publication of fuller data.

According to the findings of Chevallier & Choron (198), the feeding of vitamin A itself results in more regular increments of the bodily store of vitamin A in guinea pigs than does the feeding of carotene.

Of outstanding physiological interest are the experiments of Ralli and coworkers (199) who have studied the effect of the daily administration of carotene upon the blood carotene of normal and diabetic people, over periods of from one to four months. A given carotene intake was found to cause a greater rise of blood carotene in diabetics than in normal persons, which is interpreted to indicate a diminished power of conversion of carotene to vitamin A in diabetics.

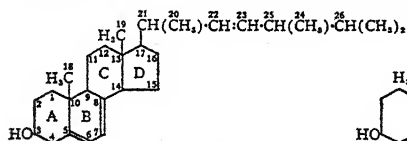
On the vexed problem of the relation of vitamin A to infectious disease, Oelrichs (200) offers further evidence from observations upon rats fed at different levels of vitamin-A intake. Proto (201) reports that direct application of vitamin A is helpful to the healing of wounds.

THE VITAMINS D

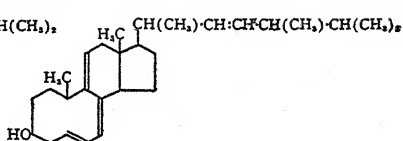
Structure of calciferol (vitamin D₂).—The term "calciferol" is here applied to that form of vitamin D (designated as vitamin D₂ by the German workers) which has been isolated from irradiated ergosterol. Although calciferol has the same empirical formula as ergosterol, C₂₈H₄₈OH, it has repeatedly been shown (202 to 205) to contain four ethylene linkages whereas ergosterol contains only three. To account for this the proposed structural formulae for calciferol represent it as a tricyclic compound in contrast with ergosterol, which is tetracyclic. Basing their suggestion partly on the work of Bernal & Crowfoot (206), who deduced from x-ray crystallographic measurements that the molecular length of ergosterol is essentially unaltered by the intramolecular changes accompanying irradiation, Rosenheim & King (207) proposed a formula for calciferol (VIII) in which the

C₅-C₁₀ linkage of ergosterol has been broken. On the other hand, Windaus (203, 208) and Heilbron (205, 209) and their associates have amassed considerable chemical evidence supporting formula IX for calciferol. Studying various degradation products of the 22-dihydrocalciferol-maleic-acid-anhydride addition complex, Windaus & Thiele (203) concluded that the latter must be represented as X. If no rearrangement has occurred during the rather drastic pyrogenic addition reaction, calciferol should be as in formula IX. The fact that ozonization of calciferol gives formaldehyde (205), and permanganate oxidation gives formic acid (205) [in yields of 20 to 30 per cent of the theory (208)], although highly suggestive of the presence of an exocyclic methylene group, is nevertheless not conclusive proof of this, since small yields of these same products may also be derived from ergosterol (208).

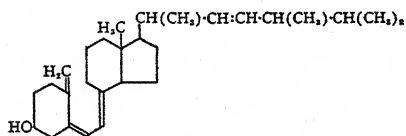
As further evidence for the rupture of the C₉-C₁₀ linkage in calciferol, and for the existence of the double bonds at C₅:C₆ and C₇:C₈, the formation of XI (205) and XII (208) from calciferol by oxidation, and of XIII (205, 208) by ozonization, is reported.



Ergosterol



VIII. Calciferol (Rosenheim & King)



irradiation into products with the calcification-promoting properties ascribed to "vitamin D": ergosterol, 22-dihydroergosterol (210, 211), 7-dehydrocholesterol (212 to 215), 7-dehydrositosterol (216, 217), and 7-hydroxycholesterol (211, 216). The first four mentioned differ from one another only in the side-chain;⁴ they show the four characteristic ultraviolet-absorption maxima of ergosterol said to be due to the conjugated double-bond system in ring B; they are presumed to yield forms of vitamin D which are alike except for the side-chain. 7-Hydroxycholesterol, however, which has no double bond in the C₇:C₈ position, shows an entirely different absorption spectrum, consisting of one rather wide band in place of the usual four and general absorption in the far ultraviolet (216). Of the vitamins D derived from these five provitamins by irradiation, that from ergosterol (calciferol, vitamin D₂) and that from 7-dehydrocholesterol (215) have been isolated. The latter, which the German workers designate as vitamin D₃ and to which they assign the structural formula XIV analogous to that proposed for calciferol (IX), appears to be somewhat less potent than calciferol. It possesses about 24,000 International units of D value per milligram, as compared with 40,000 per mg. of calciferol. Preliminary indications are that the forms of vitamin D obtained from 22-dihydroergosterol and from 7-dehydrositosterol may also show less activity than calciferol for the rat, the crude irradiation mixture from the former being roughly one-third (211) and that from the latter roughly one-fortieth (217) as active as the crude irradiation mixture of ergosterol so treated.

Elsewhere⁵ it has been shown that the question of the possible identity of two or more forms of vitamin D may be tested by assaying them with both rats and chicks and comparing the relative efficacy of the respective forms in the two species. By use of the recently defined and carefully tested technique of Massengale & Bills (219), the probable error of the determination with chicks may be reduced to less than 10 per cent of the unitage found, thus adding confidence in the validity of the deductions regarding chemical identity based on comparative assays in the two species. In the same laboratory (214, 216), it was found that the relative efficacy of irradiated 7-dehydrocholes-

⁴ An interesting observation is that 7-dehydrostigmasterol, which shows the same spectral absorption and which, according to present views of structure, differs from ergosterol only in having an ethyl group in place of the methyl at C₂₄, on irradiation develops little or no antirachitic potency (218).

⁵ Cf. *Ann. Rev. Biochem.*, 5, 386-391 (1936).

terol and that of irradiated crude (spinal cord) cholesterol were nearly identical, suggesting that 7-dehydrocholesterol may be the main provitamin present in "cholesterol" of this origin. Similar findings were reported simultaneously by Koch & Koch (213). Boer *et al.* (220) describe the actual isolation of 7-dehydrocholesterol from a cholesterol of unspecified source and uniquely high (4.5 per cent) provitamin content; but Windaus & Stange (221) advise against regarding this compound as the sole, or even the main, provitamin in all sterols of animal origin, citing in evidence their own isolation of ergosterol from the "cholesterol" of Chinese eggs.

The small amount of residual provitamin which resists the treatments employed to remove the main provitamin (7-dehydrocholesterol ?) from crude cholesterol has been found by Hathaway & Lobb (222) to differ from the latter, and also from the provitamin derived from cholesterol by heat-treatment, in giving rise to an antirachitic factor of relatively low efficacy for the chick. The provitamin of heated purified cholesterol was obtained by Koch & Koch (213) free from the typical absorption peaks characteristic of the known provitamins which have $C_5:C_6$ and $C_7:C_8$ double bonds. Their preparations showed, instead, a rather wide absorption band, the height of which roughly paralleled the provitamin-D potency. The similarity of absorption behavior to that of 7-hydroxycholesterol leads Bills (216) to suggest that the provitamin developed by heating cholesterol (with incidental oxidation) may be 7-hydroxycholesterol or some similar substance.

McDonald (211) found that addition of hydrogen to the $C_{22}:C_{23}$ double bond of ergosterol increased by several times the efficacy of the irradiation product for the chick as compared with the rat.

The relative efficacy of the irradiation products of the other identified provitamins D, 7-dehydrositosterol and 7-hydroxycholesterol, has not yet been reported from laboratories in which the highly refined rat-chick method of assay is in use; but it is of interest to note that Grab (223), using an admittedly much less accurate method but one which has given results in substantial agreement with those above quoted for irradiated 22-dihydroergosterol and irradiated 7-dehydrocholesterol, reports that more than thirteen times as many rat units of irradiated 7-dehydrositosterol as of a concentrate from tuna-liver oil are required for equal protection in the chick.

Although, in broadly general terms, it appears that the provitamins D of animal origin give rise to vitamins of relatively high, and

those of plant origin, to vitamins of relatively low effectiveness for the chick (224 to 226), it will not be surprising if application of the more exact techniques now coming into use reveals quantitative differences in this respect among different animal and among different plant materials showing provitamin activity. Thus Koch & Koch (213) report that the vitamin D of irradiated (corn-oil) phytosterol is several times more effective (rat unit for rat unit) in the chick than the vitamin D of irradiated ergosterol: a finding in contrast with the suggestion of Bethke, Record & Wilder (224) that there is no taxonomic difference between the provitamin D of higher plants and that (ergosterol) of plants of lower botanical order, such as yeasts and molds.

Kinetics of the activation process.—Hoffman & Daniels (227) confirm earlier reports that ergosterol acquires antirachitic properties when bombarded with high-voltage cathode rays. They compute that, under their conditions, one high-velocity electron produces less than one molecule of vitamin D but decomposes about 80 ergosterol molecules. The potency which may be developed is consequently considerably less than when ultraviolet light is the source of activating energy.

Rider, Sperti, and coworkers (228) hold that irradiation of ergosterol gives a much purer product of higher antirachitic value when a selected portion of the ultraviolet of longer wave length is used "with the exclusion of shorter wavelengths which may form undesired products."

Haman & Steenbock (229) found that the development of one International unit of vitamin-D activity from ergosterol required the absorption of 900 ergs of ultraviolet energy (of activating wave lengths), whereas, to develop one Steenbock unit, 3000 ergs were absorbed. Thus the comparison of the energy equivalents of the two units by these investigators agrees with their findings by biological assay, viz., that one Steenbock unit of vitamin-D activity is equivalent to 3.33 International units.

The effects of different wave lengths on the development of unpleasant flavor in irradiated milk have also been studied, by Weckel, Jackson, Haman & Steenbock (230), in relation to the antirachitic potency produced.

Determination.—Halden & Tzoni (231, 232) have described a colorimetric method for the estimation of vitamin D based on the development of a violet color with pyrogallol, with anhydrous alumi-

num chloride as condensing agent. The test requires preliminary removal of fats, fatty acids, and vitamin A and related products. Under these conditions, phytosterol, cholesterol, ergosterol, and lumisterol are said to give no color; suprasterol II gives a slight coloration which is, however, much fainter than that given by calciferol.

Another color reaction, given by vitamin D₂, vitamin D₃, and tachysterol, which has been applied quantitatively by Brockmann & Chen (233), is the development of an orange-yellow color (showing sharp absorption band at 500 mμ) with antimony trichloride in chloroform; alcohol must be absent. Vitamin A interferes appreciably only when its concentration is six times that of vitamin D. Cholesterilene interferes if present in twelve times the concentration of vitamin D, while cholesterol, sitosterol, ergosterol, 7-dehydrocholesterol, ergosterol B₈, lumisterol, suprasterols I and II, and isopyrovitamin were found to affect the determination only if occurring in amounts exceeding by forty to 400 times the vitamin D present.

For recent studies on the biological assay for vitamin-D value see (219, 234 to 241).

Vitamins D in nature.—There is also increasing evidence of multiplicity with regard to the vitamins D which occur preformed in nature, notably in the fish oils but also in egg yolk, milk, and other sources. The evidence of Bills for the existence of two or more forms (in mixture) of vitamin D in fish oils, based on the large differences among the oils of different species in their relative effectiveness for rat and chick, has now been reported in detail (214). The finding that there are large differences among the species classified as tunas with regard to the relative effectiveness of their vitamin-D mixture for the chick explains the apparent discrepancy between Bills' earlier observation of a very low efficacy ratio in certain species of tuna and the reports of Dols (242) and Rygh (243) that tuna-liver oil compares favorably with cod-liver oil in this respect. Apparently the New England and European tunas are much like the cod, the California and Oriental [see also Black and Sassaman (244)] species, definitely inferior, in relative effectiveness for the chick.

Brockmann (245), at Göttingen, has isolated a "natural" vitamin D from tuna-liver oil by use of a chromatographic adsorption technique. He reports it to be identical with vitamin D₃ (XIV) prepared by the irradiation of 7-dehydrocholesterol (cf. p. 358). This product is described as giving an allophanate of m.p. 173°–174° and a dinitrobenzoate of m.p. 128°–129°; it shows the characteristic absorption

maximum at 265 m μ observed with calciferol. What appears to be essentially the same product, although of somewhat higher potency (30,000 International units per mg.), was obtained from tuna-liver oil by Simons & Zucker (246), using a different procedure. On the other hand, Haslewood & Drummond (247) report an antirachitic substance concentrated from tuna-liver oil (to a potency of 10,000 to 20,000 International units per mg.) which preliminary observations indicate may not be the same as Brockmann's vitamin D₃.

Vitamins D in relation to rickets and to growth.—The fact that Thomas & Steenbock (248) find rolled oats, patent flour, whole wheat, polished rice, and yellow maize all to be very similar in their relation to rickets, affords additional justification for our common practice of regarding rickets as essentially due to dietary deficiencies. If there is an actively rachitogenic "toxamin" in food it would appear to be so widely and evenly distributed among cereals and their mill products as to be an essentially constant factor and rarely, if ever, the determining factor in the rickets problem.

In three independent contributions, Drake, Tisdall, and Brown have reported excellent results in the prevention of moderate or marked rickets by the use of 10 to 40 ounces daily of irradiated fresh milk which was "found to contain 94 International vitamin D units (35 Steenbock units) per 20 ounces" (249), also by the feeding of irradiated evaporated milk (250), and by the daily administration of 500 International vitamin-D units in the form of irradiated yeast (251).

The effect of various factors on the normal vitamin-D value of milk has been studied (252 to 254). The work of Bechtel *et al.* (255) indicates that the vegetable vitamin D received by the cow in such feeds as corn silage may be of greater importance in the problem than has hitherto been recognized. Wendt (256) adds to the well-known fact that the chick is less responsive than the rat to irradiated ergosterol the further suggestion that it is more responsive to the vitamin D of green feeds. He reasons that the vitamin D of green fodders, reaching people by way of cow's milk, is also, at least in such regions as northern Finland, an important factor in human nutrition. A special study of the vitamin-D requirements of chickens has been reported by Fraps and coworkers (257). The problem of the dietary control of the vitamin-D value of the egg, and its effect on the chick, has been investigated in several laboratories (258 to 260).

Jeans (261) thinks it still possible that, exclusive of purely labora-

tory products, there may be only two vitamins D: one of animal origin (typified by fish-liver oils and their concentrates, egg yolk, and ordinary milk fat) and one of vegetable origin (including yeast products). He then concludes that "animal source vitamin D milk with 135 rat units (U.S.P.) to the quart will prevent rickets, but this amount of vitamin D approaches closely the minimum effective level." Jeans holds further that mere prevention of rickets does not provide for the best growth of infants with retentions of calcium and phosphorus as great as are desirable. (Such optimal retentions of course depend largely upon the liberality of the calcium intake also.) The vitamin-D intake which is optimal for growth and development "is not known." Jeans finds also that, "vegetable source vitamin D has not been used in a manner which would determine directly the minimum rickets-preventive dose or the amount that permits good growth and retentions." Such evidence as was available seemed to Jeans to suggest that when the vitamin-D re-enforcement of milk is of vegetable origin about 50 per cent more rat units should be employed than when it is of animal origin. While Jeans has thus analyzed and interpreted the previously existing evidence, results of further research have also appeared. Eliot, Nelson, Barnes, and co-workers (262) have reported their extensive study among Detroit infants of the comparative value of cod-liver oil, viosterol, and vitamin-D milks in the prevention of rickets, and of certain factors influencing their efficacy. Unit for unit, they found no consistent difference between cod-liver oil and viosterol, while vitamin-D milk gave distinctly superior results, especially at the higher (400 unit) levels. They also conclude *inter alia* that:

The results of this study have demonstrated the necessity of considering such factors as color, sex, rate of growth in length, period of observation, and interval between examinations as basic when attempting to evaluate the efficacy of certain types of antirachitic substances administered at different levels in the prevention of rickets.

Their conclusion that vitamin D is more effective when given in the form of milk than otherwise is confirmed by Supplee *et al.* (263) who postulate a specific relationship of lactalbumin to the greater efficacy of vitamin D when administered in milk than in an oil vehicle.

Stearns, Jeans & Vandecar (264) find that maximal linear growth requires a larger intake of vitamin D than is needed for the prevention of rickets. Such constructive quantitative studies, reaching beyond the immediate question of the prevention of nutritional de-

ficiency to the larger but more elusive problem of the level of intake which induces the best results, both during development and in subsequent sectors of the life cycle, are doubtless of the most far-reaching importance and will, we may hope, be increasingly prominent in the advances to be registered in the coming years.

VITAMIN E

Evans, Emerson & Emerson (265) report the isolation from the non-sterol unsaponifiable fraction of wheat-germ oil of crystalline allophanates of two alcohols showing vitamin-E activity. One of these alcohols, designated by them as α -tocopherol, was found to show strong absorption at 2980 Å, and a slightly less intense maximum at 2920 Å; it gave an allophanate (m.p. 158–160° C.) and a *p*-nitrophenylurethane (m.p. 129–131° C.), elementary analyses of which suggested the empirical formula $C_{29}H_{50}O_2$ for the alcohol. α -Tocopherol, fed in a single dose of 3 mg., enabled female rats on a vitamin-E-deficient diet to produce litters "quite regularly." The second alcohol isolated gave an allophanate melting at 138°, and showed less vitamin-E potency than α -tocopherol, with which it appeared to be isomeric. A derivative of α -tocopherol (formed by boiling with methyl alcoholic silver nitrate) was characterized by absorption bands at 2710 and 2620 Å, and showed some vitamin-E activity. The same authors later (266) reported the isolation from cottonseed oil of an alcohol apparently identical with α -tocopherol and of a second biologically active alcohol giving an allophanate melting at 134 to 135° C.

The effect of vitamin-E deficiency on young growing rats was studied by Olcott & Mattill (267) who observed good growth in animals fed from weaning on a diet rigorously freed of vitamin E. Supplementation with vitamin-E concentrates was without effect on the growth rate of females and increased but slightly that of males. Except for complete sterility of both sexes no deficiency symptoms, such as the paresis described by Ringsted (268), were noted in thirty-two weeks.

VITAMIN K

The requirement of chicks for a fat-soluble dietary factor distinct from vitamins A, D, and E, was demonstrated by Dam (269) and Schønheyder (270) and by Almquist & Stokstad (271). Deficiency of this factor is characterized by subcutaneous, intramuscular,

and abdominal hemorrhages, and by anemia.⁶ Similar symptoms have also been produced in ducks and geese (274). The Danish investigators propose the designation "vitamin K" (*Koagulations-Vitamin*) for this factor, having noted a prolonged coagulation time of the blood in 87 per cent of the animals showing deficiency symptoms (269, 270, 275).

The failure to clot properly is apparently due to a reduced content of prothrombin in avitaminous blood (276). Vitamin K reacts negatively in direct tests for prothrombin activity but preliminary observations indicate that prothrombin, precipitated from normal chick blood by the acetic acid technique of Mellanby, retains vitamin-K activity even after repeated washings with acetone and ether. The possibility is considered that "vitamin K, or a derivative thereof, might be present in the prothrombin as a prosthetic group which is held in firm combination with the rest of the molecule" (276).

Neither normal nor avitaminous chick plasma obtained by cannulation of a blood vessel will clot without the addition of a clotting agent (such as may be prepared from embryonic or lung tissue); but the concentration of clotting agent necessary to induce coagulation of normal plasma within a given time is very much less than that required under the same conditions by plasma from K-avitaminous chicks. Schønheyder (275, 277) has utilized this property in a curative technique of assay for the antihemorrhagic factor; this has replaced the earlier preventive method in more recent Danish investigations on the distribution and chemical properties of vitamin K.

Almquist & Stokstad (278) have demonstrated the transfer of the antihemorrhagic factor from hen to chick through the egg yolk; they obtained indications of some synthesis of this factor in the intestinal tract of chicks receiving none in the diet.

The vitamin has been found in hog-liver fat, vegetables (notably the leafy varieties), and to a lesser extent in many cereals. It occurs in the easily soluble non-sterol fraction of the unsaponifiable matter, but its activity appears to be considerably enhanced by the presence of the fatty acid fraction (279). The antihemorrhagic factor is alkali-labile, relatively stable to heat, (visible) light, and standing; it distills *in vacuo* (280, 281). By distillation of a concentrate prepared

⁶ Erosions of the lining of the gizzard were also frequently observed, but these have since been attributed to lack of another fat-soluble factor which differs quantitatively from the antihemorrhagic factor in its natural distribution; unlike the latter it follows the saponifiable fraction (272). But see also (273).

from alfalfa (282), Almquist obtained a yellow viscous oil of such potency that 0.5 mg. per kg. of diet protected chicks from hemorrhagic manifestations (281).

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NUTRITION (ENERGY METABOLISM)*

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FASTING CATABOLISM

Human basal metabolism.—Benedict (1) observed that his own basal metabolism was remarkably constant from day to day and that it was increased by an emotional disturbance. The basal metabolism of women beyond sixty years of age approximates 1000 kcal.¹ per day per person irrespective of body size unless grossly over or under-weight [average deviation ± 7.1 per cent as compared with ± 6.8 per cent on the basis of body surface (2)]. The basal metabolism of elderly Japanese women does not materially differ from that of Western women. The basal metabolism of young East Indian women is, however, 20 per cent below that of Western women. Mason & Benedict (3) found that the difference is not a matter of the degree of relaxation, since sleep lowered the basal metabolism of both types of women to the same extent (10 per cent). One-fourth of the difference may be accounted for by acclimatization since Western women, after moving to the tropics, experienced a decrease in their metabolic rate of 5.1 per cent, and Indian women an increase in metabolism of 4.8 per cent after moving from the tropics to cold zones (4). Turner & Benedict (5) showed that the basal metabolism of oriental college women living in the United States on an American diet was 12 per cent below the standard for Western women, giving further proof that the lower metabolism of East Indian women is a racial characteristic.

Rabinowitch & Smith (6) studied the metabolic behavior of Eskimos in the Canadian Eastern Arctic and found that their basal metabolism was 26 per cent above the Aub and Du Bois standard. The authors relate these high basal metabolic rates to the constant stimulation of cold weather tending to produce a higher muscular tone in the Eskimos than is found in people of temperate zones.

The contrast to the behavior of Eskimos is that of Australian

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¹ 1 kcal. = 1 kilogram calorie = 1 calorie.

aborigines whose metabolism has been investigated by Hicks and coworkers (7). In spite of exposure to severe cold in naked condition the Australian aborigines in the neighborhood of Mount Liebig have a metabolism quite similar to that of Western Europeans or Americans of comparable size. The investigators explain their paradoxical results by postulating a high development of vasomotor control in the skin of the Australian aborigines which enables them to decrease the blood flow to the skin considerably, thereby decreasing the heat conductivity of the skin and with it their surface temperature and consequently their rate of heat loss.

Liberson (8) made a statistical study of the metabolism of close to 700 obese women nineteen to sixty-one years old. As an index of classification of obesity he used the quotient: $(\text{weight})/(\text{height}-105 \text{ cm.})$. This index seems to be unfortunately chosen because it depends on the size of the individual, as such, aside from the degree of obesity. The size among the experimental subjects may have been so equalized that the choice of this index may not have caused serious errors in the results, but it would be preferable in any work of this kind to choose the indices dimensionally correct, for example $(\text{weight}^{1/3})/(\text{height}-105)$. The basal metabolism of obese women is not lower, on the average, than the basal metabolism of normal women with the same surface area.

Liberson suggests the use of the square root of body weight, as characteristic of body size (Dryer), for the prediction of metabolism. Whatever may be the advantage of using the square root of weight instead of its $2/3$ power as a measure of body size in studying the metabolism of obese women, it is evident that if animals of widely different sizes are compared the square root of weight certainly cannot lead to a rational unit of surface area for similarly built animals. The results of comparisons between animals of greatly different size indicate, moreover, that the metabolism is more nearly related to the $3/4$ than to the $2/3$ power of body weight (9, 10).

Boothby and coworkers (11) report results of respiration trials on 639 males and 828 females. The subjects, selected out of a series of experiments on over 80,000 individuals, were normal human beings. The basal metabolism was expressed as calories per square meter of the Du Bois surface. The basal metabolism of the males decreased from 53.0 kcal. per sq. m. per hour at the age of six years to 35.3 kcal per sq. m. per hour at the age of sixty-four years. The basal metabolism of the females was 50.5 kcal. per sq. m. per hour at the age of

six years and dropped to 32.5 kcal. per sq. m. per hour at the age of sixty-four years. The coefficient of variability of the basal metabolism of normal human subjects according to the figures of Boothby and coworkers amounts to approximately ± 6 per cent.

McKittrick (12) concluded, from metabolism trials with 100 normal college-women students at the University of Wyoming, that the metabolism at high altitudes (7148 feet) is higher than at sea level. The basal metabolism of comparable women is 35.6 kcal. per hour per sq. m. of body surface in Wyoming as compared with 32.6 kcal. in Oklahoma and 33.4 kcal. in Florida.

Herrington (13) studied the effect of respiration of ionized air on various physiological functions of healthy young men and came to the conclusion that the respiration of positive or negative ions (mobility 0.007 to 0.0018 cm. per sec. per volt per cm.) in a concentration of 5 to 6×10^6 ions per cc. of air produces no consistent or reliable effect on the metabolic rate, systolic or diastolic blood pressure, pulse rate, rate of respiration, oral temperature, or urine volume of healthy, apparently normal, young male subjects.

Fasting catabolism of animals.—The measurements of Benedict & Bruhn (14) indicate that the metabolism of chimpanzees (2.9 to 48 kg. body weight) follows more closely the $\frac{2}{3}$ than the $\frac{3}{4}$ power of body weight. The average fasting catabolism of these animals amounted to 980 kcal. per day per unit of size, which the authors define as 10 times the $\frac{2}{3}$ power of body weight in kilograms. This unit approximately represents the body surface in square decimeters. The unit actually used by Benedict & Bruhn seems to be 1/10 of the $\frac{2}{3}$ power of body weight in kilograms, which unit approximates surface area expressed in square meters.

In contrast to the relatively high degree of constancy of human metabolism Benedict & Ritzman (15) observed a remarkable variability in the fasting catabolism of dairy cows. In a non-lactating non-pregnant cow the catabolism during the fourth and fifth days of fasting varied up to 90 per cent. For the maintenance requirement of rabbits Compel and coworkers (16) observed a similar variability though of course not strictly comparable to that of fasting catabolism and also open to criticism because there is a question as to whether or not the maintenance requirement per kilogram of body weight of one rabbit is comparable to that of another if the size is different.

Pierce (17) found that the fasting catabolism of older sheep was decidedly influenced by their nutritive condition. It amounted to 1370

kcal. per sq. m.² when the sheep had been on pasture during rainfall which kept the grass growing, and to only 1050 kcal. per sq. m. when the pasture was dry (the standard metabolism seems to have been measured forty-eight hours after the last food). Standing increased the metabolism 8 per cent over lying, sex had no influence, and shearing did not affect the rate of metabolism. Unfortunately, the environmental temperature, which might help one to evaluate the significance of the results obtained on the sheared animals, was not given.

In an extensive study on the energy exchange of swine, Breirem (18) noted a fasting catabolism of 1980 kcal. per day per 100 kg. body weight. With a Meeh constant of 10 this result corresponds to 920 kcal. per sq. m. of body surface.

In respiration trials with rabbits Tomme & Loria (19) found that the third day of fasting is suitable for determining the fasting catabolism which amounts to 611 kcal. per sq. m. per day. The surface area was determined as $13.2 W^{2/3}$ (W = weight in kg.).

In an investigation on the Terroine-Sorg-Matter relation (endogenous nitrogen excretion/basal metabolism), Ashworth (20) discovered that during the sixth to twelfth day of low nitrogen intake growing rats, which had previously been kept on a high nitrogen level, had a higher nitrogen excretion and a lower fasting catabolism per unit weight than rats previously kept on a low nitrogen level.

Barott and coworkers (21) studied the energy metabolism and respiratory exchange of normal and deutectomized chicks between ten and one hundred hours of age. They state that respiration trials with normal chicks under five days of age cannot be accepted as measurements of the fasting catabolism because the bodies of these chicks contain various amounts of unabsorbed yolk which plays the same rôle as ingested food. The investigation was carried out in a respiration calorimeter. Normal chicks had a minimum rate of metabolism at 96° F. (35° C.). At 89° F., as well as at 101° F., their metabolism was 15 per cent higher than at 96°. The lower temperature limit for newly hatched chicks is 70° F. Their metabolism at this temperature is twice the minimum metabolism. The deutectomized chicks showed essentially the same reaction to environmental temperature as the normal chicks. The metabolism of the deutectomized chicks can be calculated from the metabolism of the normal chicks by a linear equation in which the time in hours after the removal of

² The surface was calculated as $0.121 \times W^{0.59}$ where W = weight in kg.

the yolk is a factor. The rate of absorption of the yolk was found to be independent of the nutritional requirement of the chick: the yolk was absorbed by the fasting chick with the same rate as by the fed chick. The fasting normal chick with its constant rate of yolk absorption maintained a constant rate of metabolism for four days while the deutectomized chick showed a continuous decrease during fasting. The heat production of the chicks per gram loss of weight was more than twice as great at 68° F. as it was at 107° F., indicating that at a high environmental temperature the body of the chick tends to become dryer, and at a low environmental temperature more watery, than at a medium temperature. The drying effect of high environmental temperatures has also been observed in pigeons by Dontcheff & Kayser (22) who concluded from their measurements that one-third of the extra water lost at high temperature came from the tissues and two-thirds from the excreta by way of reabsorption in the intestinal tract.

In spite of the difference in the metabolism of the deutectomized and normal chicks there was no difference in their respiratory quotients. This result is in agreement with the findings of Henry and coworkers (23) who studied the effect of fasting on the composition of the blood and the respiratory exchange in fowls. Fasting R.Q.'s as low as 0.64 were observed. In explanation it was contended that these low quotients are the result of a nitrogen metabolism peculiar to birds where uric acid instead of urea is the main form in which nitrogen is excreted. These authors showed theoretically that alanine oxidized to uric acid, carbon dioxide, and water should produce an R.Q. of 0.67 and observed that continuous feeding of coagulated egg white, casein, and fishmeal to birds resulted in R.Q.'s not higher than 0.697.

Nichita & Cretzu (24) report on metabolism studies with different races of chickens in which the fasting catabolism per unit of body weight was inversely proportional to the linear dimensions of the animal. From this statement one may conclude that the metabolism of these birds was proportional to the $\frac{2}{3}$ power of body weight or the body surface.³

³ If B = fasting catabolism, W = body weight, L = a linear dimension, and K and K' are constants, then Nichita's statement may be formulated as follows: $B/W = K/L$; since, in animals of similar build, $L = K'W^{1/3}$ it follows that $B/W = K/K'W^{1/3}$ or $B = \text{constant} \times W^{2/3}$.

An interesting result has been obtained by Smith (25) who observed that the oxygen consumption of lung fish 300 days after the last food was between 10 and 20 per cent of the oxygen consumption when fully fed.

TEMPERATURE REGULATION

Body temperature.—Benedict & Lee (26) have observed that the body temperature of elephants, measured as temperature of the urine, varies within narrow limits about an average of 35.9°C .; this is remarkably low.

Considerable work has been done recently on the measurement of the surface temperature of human beings. Hardy (27) has constructed a radiometer which allows measurement of the surface temperature without disturbing the processes of heat exchange of the skin. This radiometer measures the skin temperature with an accuracy of $\pm 0.1^{\circ}\text{C}$.; the errors of the ordinary measurements of skin temperature may be as high as ± 5 per cent. Burton (28) measures the average temperature of extended areas of the human skin by means of resistance thermometry. Laroche & Saidman (29) noted the influence of the climate on the skin temperature of human beings; they give 32.2°C . as a grand average for the temperature of the entire skin.

Variations of the body temperature of human beings were sufficient to produce differences between the results of direct and indirect calorimetry as high as 20 per cent in a new respiration calorimeter developed by Murlin & Burton (30) in which the differences between the two methods of heat determination in alcohol checks amounted to 2.9 per cent. The temperature of the human body is not adequately represented by the rectal temperature but by an average body temperature calculated as the sum of 0.65 times the rectal temperature plus 0.35 times the average surface temperature. The changes in the surface temperature are, on the average, three to four times as great as the corresponding changes in the rectal temperature [Burton (31)]. After a meal, heat is first stored in the body and raises the rectal as well as the surface temperature. The rise of the surface temperature amounts to 1°C ., with a simultaneous rise of the rectal temperature of 0.25°C . (32). The "thermal circulation index," (surface temperature—room temperature)/(rectal temperature—surface temperature), indicates a general rise in heat circulation after a meal. This rise of

heat circulation is correlated with an increase in the rate of blood circulation. The increase in rate of circulation after a meal is general; there is no evidence of a withdrawal of blood from the outer parts of the body to provide the increased supply for the viscera.

Heat loss.—Studies on the partition of heat loss have recently been intensified by the group of biophysicists at Yale. Winslow and co-workers (33) constructed a copper booth surrounding the experimental subject, which enables the experimenters to vary the radiation climate independently from the conduction and convection climate. They are able to produce a difference of 40° C. between the temperature of the ambient air and the mean radiant wall temperature. A mathematical test enables them to discover possible errors in their measurements and assures the range of conditions within which their results are reliable [Gagge (34)]. In the series of experiments so far reported, with radiation temperatures from 35° to 56° C., air temperature varying from 33° to 17° C., and a variation of heat loss by evaporation from 27 to 184 kcal. per hour, Winslow and coworkers (35) find a probable error between two ways of determining the convectional heat loss of less than 4 per cent of the metabolism. They compute a "radiating surface" of the human body which is 75 per cent of the surface calculated from weight and height according to Du Bois.

The human skin according to Hardy's measurements (27) radiates like a black body. This behavior is independent of the visible color of the skin. The pressure of water vapor and carbon dioxide in the layers of air near the skin have no influence on its behavior as a radiator. In further experiments Hardy & Muschenheim (36) reached the conclusion that the emission spectrum of human skin is that of a black body and also that the infrared transmission and reflection spectra obtained with human skin are equal to those of a black body.

Christiansen & Larsen (37) conclude from their measurements, in contrast to Hardy's results, that human skin has, in general, a smaller radiation than a black body, and that the radiation constant depends on the blood content of the skin (38); the radiation constant increases steadily from anemic through normal to hyperemic skin and rises with increase of temperature (39).

Witz (40) observed that the heat transmission coefficient (on the basis of Fourier's law of heat flow) of the human skin varies greatly with temperature variations, thus confirming results obtained by the reviewer (10).

Chemical temperature regulation.—The increase of metabolism with decrease of environmental temperature which takes place at low environmental temperatures is known as chemical temperature regulation. It is to be contrasted with the physical temperature regulation which is in operation above the so-called critical temperature of the environment, when the animal is to be protected against overheating. The animals prefer the region above the critical temperature (*Behaglichkeitsgrenze*). Their choice of environmental temperatures seems to be a genetic characteristic [Herter (41)].

The fasting catabolism of homotherms depends not only on the temperature at which the metabolism is measured, but also on the temperature under which the animals have been kept prior to the metabolism test. Gelineo (42) measured the metabolism of rats at a temperature of 31.5° C. and then exposed the rats to 36° C. during forty-five minutes, thus raising the body temperature from 36.5 to 39.5° C. During the next nineteen minutes, when the rat was kept at 23° C., its body temperature returned to normal but its metabolism (tested again at 31.5° C.) was decreased 12 per cent. This decrease of the metabolism by hyperthermia of short duration occurred only in rats that had been adapted previously to a low environmental temperature. Rats which had lived for a month at 30 to 32° C. did not exhibit any change; the metabolic rate remained at 517 kcal. per sq. m. of body surface. Rats kept for a month at 16 to 20° C. had a metabolism of 676 kcal. and rats kept for three weeks at 5 to 10° C. produced 859 kcal. (measured at a temperature slightly above 30°). Gelineo calls the fasting metabolism of the heat-adapted rats (517 kcal.) the "physiological energy" and the increase in fasting metabolism found in rats which had been kept at a low environmental temperature the "thermic adaptation energy." Hyperthermia has no effect on the "physiological energy" of the fasting catabolism. The increased fasting catabolism of rats adapted to low temperatures is in harmony with the results on Eskimos already mentioned (page 375).

Physical temperature regulation.—Kleiber & Regan (43) studied the influence of environmental temperature on the respiratory frequency and the rate of ventilation of dairy cows. The respiratory frequency of cows increases with increasing environmental temperature above 10° C. This increase of the respiratory frequency with temperature follows closely the Arrhenius equation for the influence of temperature on chemical reactions. The effect is related mainly to the temperature of the air surrounding the animal's body; the tem-

perature of the inspired air has but a slight effect. The depth of respiration is decreased at high environmental temperature as the frequency is increased, thus securing an increased dead space which probably prevents an over-ventilation of the *alveoli* and yet permits full use of the upper parts of the respiratory tract for water evaporation.

Lee & Mulder (44) observed in man at a high environmental temperature an increase in depth rather than in frequency of respiration which is contrary to the behavior of the cow and seems to demonstrate that man is not as well adapted to this type of physical temperature regulation as is the cow. Pigeons behave similarly to cows, increasing the frequency and decreasing the depth of respiration at high environmental temperatures. Saalfeld (45), who made this observation, reports that the center for polypnea (*Hachelzentrum*) is directly affected by temperature and that local cooling of this center prevents polypnea even though the body temperature is raised. It may be mentioned in this connection that Thauer (46) discovered in rabbits that peripheral nerves are able to regulate the body temperature to a considerable extent after the influence of the central nervous system is excluded.

Insensible perspiration.—Mitchell & Hamilton (47) have worked out an equation for the calculation of the metabolism of cattle from insensible perspiration. The equation contains the ratio of heat lost by evaporation to total heat loss. Since the evaporation of water is an important part of the physical temperature regulation this ratio is bound to vary greatly with the thermal condition of the animal, as was shown for man by Rubner (48). That is why at higher planes of nutrition relatively larger proportions of heat are lost as heat of evaporation, as was pointed out by Kriss (49), who observed that the heat of evaporation with one animal rose from 22.2 per cent to 46.2 per cent of the total heat given off when the ration was more than doubled. In order to keep the evaporation in a constant proportion to total heat loss it would be necessary to vary the environmental temperature according to the plane of nutrition. It is questionable whether the measurement of the insensible loss will ever be a reliable substitute for the measurement of metabolism. However, the observation of Mitchell & Hamilton, that the ratio of heat of vaporization to total heat loss varies greatly among different steers but is remarkably constant for any given steer, is of interest. It is in harmony with the conclusion reached by Witz (40) that in each species each individual has its own particular method of heat regulation.

CALORIGENIC ACTION

Terminology.—The love of German authors for the word *dynamisch* led Rubner⁴ and Kellner to use it in conflicting ways. The heat increment of the food (Armsby) results from the "specific dynamic action" (Rubner) and the other part of the metabolizable food energy (Armsby's net energy) is "dynamic energy" (Kellner). The term "calorigenic action" (Mitchell; Luck) appears to be a good way out of the linguistic trouble. "Calorigenic" seems preferable to "thermogenic" as used by Hamilton (50) since thermogenic should be applied to indicate an increase in temperature. We use a thermometer for measuring temperatures but a calorimeter for measuring heat quantities. A drug such as dinitrophenol may have a thermogenic action (raising the body temperature) as well as a calorigenic action (raising the heat production). The same is true for the thyroid hormone, the thermogenic action of which, particularly with respect to the skin temperature, has recently been studied by Laroche and co-workers (51).

Calorigenic action of nutrients.—Borsook (52) has summarized recently his theory of the calorigenic action of proteins and amino acids. A constant part of this effect includes 4 kcal. per gm. of nitrogen metabolized, resulting from oxidative deamination, 4 kcal. used for synthesis of urea from ammonia and 1 or 2 (or less) kcal. used for the excretion of urea. Rajzman (53) found that the renal work is insignificant. A variable part of the calorigenic effect comes from the metabolism of the nitrogen-free part of the proteins.

The calorigenic action of protein should not be related to the ingested but to the metabolized protein [Wilhelmj (54)]. The calorigenic action therefore depends on the nutritive level of the food; it is minimal with a completely balanced ration [Mitchell (55)]. This idea has been expressed by Møllgaard (56) who found that the utilization of food energy for milk production is optimal (calorigenic action minimal) when the ratio of protein energy to energy in the nitrogen-free nutrients in the ration approximates the corresponding ratio in milk, i.e., is between 0.14 and 0.23. The same idea is also contained in Hansson's (57) distinction between the relative nutritive value of protein for fattening and for milk production (1 kg. protein \equiv 0.94 kg. starch value for fattening; but 1 kg. protein \equiv 1.43

⁴ Rubner also speaks of isodynamic nutritive values when he refers to heat and not to work and when, therefore, equicaloric would have been a more precise term.

kg. starch value for milk production). The factor 1.43 is based on the assumption that that part of the food protein which is employed for milk production does not produce a greater heat increment than fat or carbohydrate during their conversion to the nitrogen-free constituents of milk.

Hamilton (50) found in growing rats that the calorogenic action of the ration decreased as the percentage of protein in the ration increased from 4 to 16 per cent. Since protein has a far higher calorogenic action than non-protein nutrients this result seems paradoxical but it is in harmony with the idea that utilization of food energy is optimal in rations balanced for special nutritional requirements.

Practically the same result was reported by Forbes and collaborators (58), who found in forty-eight albino rats, kept for ten weeks on four different planes of protein intake (10, 15, 20, and 25 per cent of the ration) at equicaloric food intake, that increases in the protein content of the diet increased the gain in weight, increased the weight efficiency (gain per unit of food consumed), and increased the digestibility of protein, but did not materially affect the metabolizability of the food energy. The increase in protein in the equicaloric rations did not affect the basal heat production of the rats but diminished their total heat production when fed. This result at constant metabolizability means, therefore, a decrease in the calorogenic action of the food with increased protein content, in line with the observation of Hamilton. A well-defined calorogenic action of protein can only be expected in animals which neither gain in body protein nor excrete protein in milk. But this calorogenic action then would lead to only a minimal nutritive value for the food protein, its energy value for producing body fat.

Instead of indicating a loss of food energy the calorogenic action may be an index for the therapeutic action (stimulation of metabolism) of food materials. An investigation of the calorogenic action of grape juice was carried out from this point of view by Epstein and coworkers (59). These authors observed that the calorogenic action of grape juice considerably excels the calorogenic action of the monosaccharides contained in the juice. The ingestion of 200 cc. of white pasteurized grape juice increased the oxygen consumption of human subjects, twelve hours after the last food, 23 per cent in the first half hour, 20 per cent after one hour, 11 per cent after one and one-half hours, and 1.5 per cent after two hours.

Murlin and coworkers (60) investigated the calorogenic action of

butterfat in man during the fourth or fifth day of an all-cream diet. The calorogenic effect amounted to 4.74 per cent of the energy contained in the fat fed. The calorogenic action of fat was not parallel with the fat content of the blood. This result, according to the authors mentioned, indicates that Lusk's plethora theory does not completely explain the calorogenic action of fat, for which it has so far not been disputed (54).

The calorogenic action of glucose is probably connected with the synthesis of glycogen from glucose; it is as a rule greater after hepatectomy (54). This is in contrast to the calorogenic action of protein, the major part of which occurs in the liver.

Murlin and coworkers (60) have observed cases in which the calorogenic action of sugar and fat together was greater than the sum of the effect which sugar and fat would have produced if fed separately.

Calorogenic action of hormones and vitamins.—Similar to the behavior of a combination of sugar and fat is that of a combination of prolactin and thyrotropic hormone in producing a synergistic action on oxygen consumption which exceeds the sum of the separate effects of these hormones. Riddle and coworkers (61) who observed this relation report further that the marked calorogenic action of prolactin is unlike that of thyrotropic hormone in that it is effective independently of the thyroid gland, and that the follicle-stimulating hormone, as well as prolan, does not produce a calorogenic effect.

The calorogenic action of vitamin D in dogs disappeared when the dogs were thyroidectomized, in experiments carried out by Deutsch and coworkers (62). Thyroidectomized pigeons similarly did not increase in metabolism after treatment with dinitrophenol [Riddle & Smith (63)].

Influence of temperature on calorogenic action.—The calorogenic action depends on the environmental temperature as follows: At 0 to 5° C. there is no calorogenic action; all the heat arising from metabolism of protein is used for heat regulation (Rubner's compensation theory). Between 15 and 20° C. the constant part of the calorogenic action (heat arising from oxidative deamination, synthesis of urea, and excretion of nitrogen) is observed but the heat increment of the nitrogen-free part does not appear; there is also no calorogenic action of fat or glucose. Above 25° the full calorogenic action, including the variable fraction arising from metabolism of the nitrogen-free part, appears [Borsook (52)].

Riddle and coworkers (61) found that the calorogenic action of prolactin and thyrotropic hormone in pigeons occurred at an environmental temperature of 30° C. but disappeared at 20° C. At 14° C. there was actually a decrease in metabolism in the hormone-treated pigeons as compared with normal birds. The disappearance of the calorogenic effect at low environmental temperatures is in harmony with Rubner's compensation theory (48). (The calorogenic effect is used for heating the animal's body and thus saves body substance from decomposition.) The same effect of low temperature has been observed on the calorogenic action of dinitrophenol by Tainter (64) in rats and by Riddle & Smith (63) in pigeons.

In contradiction to Rubner's compensation theory are the results obtained on rabbits by Tomme & Missiutkina (65) who noticed at freezing temperature a calorogenic action of the food as great as at 30° C.

VITAMIN AND MINERAL DEFICIENCIES AND UTILIZATION OF FOOD ENERGY

Vitamin deficiency and food utilization.—A qualitative deficiency in a ration may lower the efficiency of food utilization by decreasing the appetite, by increasing the fasting catabolism, by decreasing the digestibility or metabolizability of the food, or by increasing its heat increment (calorogenic action).

Schoch (66) studied the food utilization of vitamin-D deficient and normal rats in a series of respiration trials. He found no difference in the fasting catabolism. The digestibility was not affected by vitamin-D deficiency and an effect on the heat increment seems also not to have been observed.

Braman and coworkers (67) carried out an extensive investigation on the influence of deficiencies in vitamins A, D, and G, on the food utilization of growing rats. These workers used the paired feeding method where the food intakes of two pair mates, one a deficient and the other a normal animal, are equal (as a rule determined by the food intake of the deficient animal whose appetite is mostly below that of the control rat). They determined the energy exchange by measuring the energy in the food, excreta, and in the bodies of the rats which were killed after the trial. The composition of the rat bodies at the start was estimated by analyzing control rats which were comparable to the experimental animals. The influence of the vitamin deficiency

on the food intake is thus eliminated; consequently a depressing influence of a deficiency can mean only an increase in the fasting catabolism or a decrease in the partial efficiency of the food (change in net energy/change in food energy) which latter may mean a decrease in digestibility, metabolizability, or an increase in heat increment.

From an experiment with vitamin-A deficiency (the experimental rats kept in only moderate vitamin-A depletion) the authors concluded that the vitamin-A supplemented diet was more palatable and produced, at equal levels of energy intake, a higher gain in weight than the vitamin-A deficient diet. However, digestibility, metabolizability of the ration, as well as heat production of the animal and its storage of energy in body substance were unaffected by the difference in the vitamin-A content of the rations. It was noted that lack of vitamin A decreased the appetite.

A vitamin-D deficient ration produced a greater heat loss by the animal than the equicaloric control ration with a supplement of vitamin D. The rats on the vitamin-D supplemented ration had a better appetite and gained more fat and energy than their pair mates on the vitamin-D deficient ration.

Deficiency of vitamin G depressed the appetite and decreased the growth rate and the storage of energy in body protein and body fat. An increase of the ratio, C/N, in the G-deficient rats indicated that lack of vitamin G leads to incomplete oxidations.

Mineral deficiency and food utilization.—Kleiber and coworkers (68) investigated the influence of phosphorus deficiency on food utilization of six beef heifers; thirty-four complete respiration trials of two weeks' duration each and sixteen tests of the fasting catabolism of two days' duration each, using a double chamber respiration apparatus (69), were run. They confirmed the observation of Riddell and coworkers (70) that phosphorus deficiency does not affect the digestibility of the food. They found, however, no influence of phosphorus deficiency on the fasting catabolism and concluded that phosphorus deficiency lowers the total efficiency of energy utilization, (energy in produced body substance)/(energy in the entire food), mainly by depressing the relative food intake of the animals and secondly by decreasing the partial efficiency, (change in energy of body substance)/(change in energy of food); this amounts to an increase of the heat increment since the digestibility is not affected.

Aubel and coworkers (71) report extensive experiments on the phosphorus requirements of growing pigs. The food intake was con-

trolled. In order to secure the food consumption of the phosphorus-deficient pigs at a desired high level the food was given in the form of a slop, thus taking advantage of the great thirst of the phosphorus-deficient animals. The increase in body weight was smaller in the phosphorus-deficient pigs than it was in the controls. The phosphorus-deficient pigs also stored less energy in their bodies, but they carried a higher percentage of fat and yielded better finished carcasses. Since the food intake was kept at the same level for the phosphorus-deficient and the control pigs the results of Aubel and coworkers indicate a depressing influence of phosphorus deficiency on the partial efficiency of energy utilization which is in harmony with the results of Kleiber and coworkers on beef heifers.

Food composition and body size.—The energy requirement of animals is proportional to their fasting catabolism and since the fasting catabolism is proportional to the $\frac{3}{4}$ power of body weight the energy requirement also is proportional to the $\frac{3}{4}$ power of body weight. The requirement for minerals and vitamins is, in some cases, in a different relation to body size from that of the energy requirement. If this is the case then the necessary concentration of some of these essential constituents in the food must be a function of body size. An example of this relation is the vitamin-A requirement per unit of food intake.

Guilbert & Hart (72) presented evidence that the vitamin-A requirement of rats and cattle follows more closely the body weight than the energy requirement. They conclude from their investigation that the minimum requirement of mammals is 20 to 30 μg . of vitamin A or carotene per kilogram of body weight. Guilbert and coworkers (73) have extended the studies on the minimum vitamin-A requirement to sheep and swine and have confirmed the earlier conclusion based on cattle and rats, that the vitamin-A requirement is proportional to body weight (20 to 30 μg . per kilogram daily). Since the energy requirement is proportional to the $\frac{3}{4}$ power of body weight then the necessary concentration of vitamin A in the food should be proportional to the fourth root of body weight.⁵

The vitamin-B requirement, unlike that of vitamin A, seems to be directly proportional to the energy metabolism [Cowgill (74)].

⁵ If V = amount of vitamin, W = body weight, U = amount of food, measured as food energy, and a and b are constants, then V/U = concentration of vitamin in the food. If, further, the vitamin requirement is proportional to body weight ($V = aW$) and the energy requirement is proportional to the $\frac{3}{4}$ power of body weight ($U = bW^{3/4}$) then $V/U = a/b \times W/W^{3/4} = \text{constant} \times W^{1/4}$.

Not enough is known at the present time about the phosphorus requirement to decide whether it follows the body weight or the metabolic body size ($\frac{3}{4}$ power of weight). There are some indications that similar relations may be found as for vitamin A. Thus Gilbruth (75) reports that phosphorus deficiency in the food is not as serious for rabbits as it is for sheep and cattle, and Marston (76) observed that sheep are less affected by phosphorus-deficient pasture than are cattle. He explains this observation partly by the fact that sheep select their food more carefully than do cattle, but also by the greater food intake of the sheep per unit body weight. Underwood & Shier (77) also state that phosphorus requirements of sheep are probably relatively low. Pica, observed in Australian sheep which eat the carcasses of rabbits but do not exhibit any particular craving for bones, was not cured by phosphate licks and was related by these authors to the dry fibrous nature of the diet rather than to its low phosphorus content.

The protein requirement seems to be nearly proportional to the energy requirement (78). There is, however, an exception: The protein requirement for growth in human beings is relatively small in proportion to the energy requirement, as compared with the protein requirement of other animals, because human growth is exceptionally slow [Terroine (79)]. The small daily production of body protein indicates that the protein concentration in human food may be much smaller than in a well-balanced ration for the growing pig since the energy requirement for maintenance for both animals is approximately equal.

EFFICIENCY OF ENERGY UTILIZATION AND BODY SIZE

Brody & Cunningham (80) studied the records of a one hundred twenty days' test on high-producing dairy cows at the World Fair in St. Louis in 1904, and concluded that the average total efficiency of milk production (energy in milk/digestible energy in total food) was 34 per cent. The partial efficiency (energy in milk/digestible energy in that part of food which is available for milk production) was approximately 60 per cent. In ordinary Experiment Station cows the total efficiency for milk production was 30 per cent; in a group of superior Holsteins in Illinois, 34 per cent. Extreme total efficiencies were found for the 1700-pound champion Holstein cow, Carnation Ormsby Butter King "Daisy," namely, 43.5 per cent, and the 700-pound champion Jersey cow, Stonehurst Patrician "Lily," namely,

47.5 per cent. A high total efficiency is related to a high food capacity. The relative food intake (digestible energy in the food/basal metabolism) for the superior Holsteins with 34 per cent efficiency was 7 while the ordinary Experiment Station cows with 30 per cent efficiency digested 5 times as much energy in food as they would have expended during rest and fasting. This recent study of Brody & Cunningham confirms the results of earlier work and with it the idea that total efficiency of milk production is essentially independent of body size.

Brody & Cunningham (81) also investigated the efficiency of horses for mechanical work and compared this efficiency with that of man and that of a tractor. The oxygen consumptions of a 1500-pound Percheron gelding and of a 600-pound Shetland pony were measured with a closed respiration apparatus connected to the animal by a nose mask while the horse was working on a treadmill. Brody & Cunningham conclude from their results that the maximal total efficiency of muscular work (energy of mechanical work performed)/(chemical energy expended by the animal during work) is approximately 25 per cent for either horse and is thus independent of body size. It is also the same for work by human beings.

Gollwitzer and coworkers (82) found with heart-lung preparations a maximum efficiency for the heart of 19 per cent; most results were between 6 and 13 per cent. Grosse-Lordemann & Müller (83) measured the work of a human being on a bicycle; their data indicate efficiencies up to 19 per cent.

A great number of data on feed-lot experiments with cattle and sheep compiled by Rochford (84) lead to the conclusion that the total weight efficiency (gain in weight/weight of food consumed) is the same for cattle and for sheep. The average daily food consumption, as well as the daily gain in weight, followed closely the $\frac{3}{4}$ power of body weight indicating that this unit of reference is suitable not only for comparing basal metabolism but also for comparing growth rates and food consumption.

The results of Brody and coworkers, as well as the data reported by Rochford, tend to strengthen the idea that, in general, efficiency of food utilization is not related to body size (85). It is of course possible that body size affects the efficiency of food utilization in particular cases, but even for those possible cases, it would be a mathematical error to express efficiency by an index which contains a term of body size. It is reasonable to express the daily production of an animal,

as well as its daily food intake, in calories per unit of body size, but it is erroneous and misleading to express efficiency as a function of body size. Energy efficiency (energy gained/energy spent) must be a dimensionless term.

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THE BIOCHEMISTRY OF MUSCLE*

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MUSCLE PROTEINS AND THE STRUCTURE OF THE FIBRILS

Following the pioneer work of Weber, Stübel, Edsall, Murali *et al.* much work has been done in the last few years on the separation and nature of the muscle proteins, and on the correlation of these chemical facts with histological and morphological data. It is now clear [cf. Bate Smith (1)] that at least four proteins are present (besides the connective tissue or stroma proteins), and in approximately the following proportions: myosin, 68 per cent; globulin X, 21 per cent; myogen, 10 per cent; and myoalbumin, 1 per cent. Some important properties used for differentiation are the isoelectric points, and the solubilities of the native and denatured proteins in water,

TABLE I
PROPERTIES OF MUSCLE PROTEINS

Isoelectric Point		Albumins		Globulins	
		Myogen	Myoalbumin	Myosin	Globulin X
		6.7	3.3	5.5	5.2
Solubility of native proteins in.....	H ₂ O	+	+	—	—
	Salts	+	+	+	+
	HCl	+	+	+	+
Solubility of denatured proteins in.....	H ₂ O	—	—		—
	Salts	—	—		—
	HCl	+	—		+

acid and salt solutions (Table I). Other properties used for comparison are double refraction of flow (shown only by myosin solu-

* Received January 23, 1937.

tions), rate of denaturation, and character of precipitates. It seems that myosin as ordinarily prepared consists of a single native protein species; myogen and myoalbumin cannot be separated in the native state but after denaturation a fairly clean separation can be made; globulin X is difficult to prepare free from denatured albumins. Knowledge of the solubilities of these fractions throws light on their state in the muscle fibre. Smith (2) has investigated the dependence of the solubility of myosin on salt concentration and on pH. He concludes that at the pH of resting muscle (about 7.2) and in the salt concentration present there (equivalent to 0.18 *M* KCl) at least 90 per cent of the myosin must be in the gel form. Muscle press-juice, which represents the sarcoplasm surrounding the fibrils and amounts to 30 per cent of the muscle weight, contains all the myogen, myoalbumin, and some globulin X. This juice is expressed at pH 5.8 to 6.2; at the pH of resting muscle it is likely that the globulin X would be completely dissolved in the sarcoplasm. No doubt the lowered pH also affects the volume relations of fibril and sarcoplasm, causing passage of water to the latter. Small amounts of phosphate ions have a peculiar effect in increasing the solubility of myosin and there is enough phosphate in press juice (owing to the breakdown of creatine-phosphate and adenylypyrophosphate) to have this dissolving effect. The fact that no myosin does appear in press-juice may indicate that the surface of the fibrils differs from the interior.

The comparison of myosin threads with muscle fibrils has been carried further [Weber (3); Noll & Weber (4)]. These threads, when first made by squirting myosin solution (1.5 per cent) into water, contain only 1 per cent of myosin and are feebly doubly refracting. On stretching (30 to 50 per cent) and drying they become strongly birefringent. When soaked in 0.1 *M* phosphate at pH 7.4 they take up water until they contain only 16 to 20 per cent of protein, i.e., an amount equivalent to the protein content of the whole muscle. The work of Stübel (5) on dependence of double refraction on the refractive index of penetrating fluids was repeated quantitatively and extended. Myosin threads, like muscle fibres, show *Stäbchendoppelbrechung* (St-Do) and *Eigendoppelbrechung* (E-Do). For muscle, the double refraction (Do) in water is 2.3×10^{-8} ; it alters, with penetration of different liquids, to a minimum when the refractive index of the liquid is 1.56. This value, which agrees with the refractive index of the dried myosin threads, is much higher than that given by Stübel. Calculated on the same protein content, the fibril has

40 per cent of the Do and 40 per cent of the E-Do of the myosin thread. If the birefringent parts of the fibril consist of the same material as the myosin thread they should occupy 40 per cent of the whole fibre volume. This seems to be the case; recent values for the relative lengths of the anisotropic and isotropic bands in frog-muscle fibrils (6) give 55:45; and, as we have seen, the fibrils constitute about 75 per cent of the muscle volume. These results are taken to mean that the anisotropic bands of the fibrils are made of myosin micellae orientated to some degree with their long axes parallel to the long axis of the fibril. The isotropic bands are not completely without birefringence, showing about 10 per cent of the activity of the anisotropic bands (7); they also must be made of myosin micellae in a less ordered arrangement. The results of Eggleton & Eggleton, showing that certain ions diffuse into only 20 to 30 per cent of the muscle volume, harmonise with the view that 75 per cent of the volume is occupied by semi-permeable fibrils (see, e.g., 8).

X-ray photographs of myosin threads were found by Boehm & Weber [see Weber (9)] to be similar to those of muscle. Worschitz (10) has also taken x-ray photographs of muscle. Isotonic contraction leads to a diminution of pattern in the photographs, indicating distortion of the micellae or a decrease in the orderliness of their arrangement [Boehm (11)]. This change was not visible with isometric contraction. Astbury & Dickinson (12) found that myosin films, made by pouring solution on to glass plates and allowing them to dry in air, give moderately good photographs, showing that the molecular chains are roughly parallel to the surface. On moistening and stretching this parallelism increases and an x-ray photograph is obtained which resembles that of α -keratin; on further stretching the typical β -keratin photograph is obtained. If an unstretched myosin film is exposed for a few seconds to steam it contracts spontaneously by about 20 per cent; this behaviour resembles the supercontraction of keratin, but is brought about more readily, suggesting that the side chain linkages are less stable in this case. The α and β keratin-like forms were demonstrated a little later (13) in frog-sartorius muscle and in the retractor of the foot of *Mytilus*; the unstretched muscle gave the α form, the stretched the β form. For a discussion of the conception of myosin as a protein "configurationally disposed towards denaturation" see Astbury *et al.* (14, 15). Mirsky (16) has studied the effect of denaturing agents on the sulphydryl groups of muscle proteins.

There is general agreement that it is the anisotropic band which shortens when the fibril contracts, the isotropic band showing little change in length. [For recent work on this subject see (17, 18).] A decrease in double refraction also occurs on contraction, even when isometric (19). Muralt regards this decrease as more probably due to change in the chemical nature of the myosin (change in E-Do) than to decrease in symmetry.

Two other effects of contraction have been further studied—the changes in volume and the increase in transparency. Meyerhof & Möhle (20) find that the volume changes cannot be entirely explained by change in molecular volumes consequent upon the known chemical reactions, although for short experiments the excess constriction observed is not great. The correctness of Meyerhof's general interpretation has been questioned by Fischer (21) in the light of experiments on the smooth muscle of *Phascolosoma* where the volume change per gm. of tension was 100 times as great as for the frog. The direction of the volume change was not constant and could be influenced by physical conditions such as initial length and tension. With frog's gastrocnemius Meyerhof's results were confirmed (22) except that under high initial tension an increase, not a decrease, in volume was observed. With sartorius and semimembranosus not very high tensions were needed to get this effect, and the question arises as to whether the initial tension alters the qualitative nature of the chemical changes, or whether the volume changes are governed, at least in part, by physical changes inside the fibres.

Baeyer & Muralt (23) and Muralt (24) showed that an increase in transparency accompanies isometric contraction; aerobic recovery is accompanied by a complete return to normal, anaerobic recovery by a partial return. In muscles poisoned by iodoacetic acid there is no return anaerobically. These facts led to the suggestion that the increase of transparency depends on a change in the nature of the muscle proteins, which in turn depends on the breakdown of creatine-phosphate. Lactic acid formation seems to have no important effect on the optical behaviour of muscle. The increase in transparency has also been observed by Nicolai (25). Both Nicolai and Sandow (26) studied the variations in diffraction pattern on contraction.

The immediate cause of the change in length and thickness of the myosin micellae is not clear. It may be due to the dehydrating effect upon the micellae of some ionic change in the solution surrounding the fibrils, the removal of water leaving the protein chains free

to crumple in some way [Bernal (27)]. That contraction is accompanied by dehydration of muscle proteins is indicated by the decrease of their solubility in certain salt solutions on fatigue [Deuticke (28)]. The effect of ions on the shape and size of myosin micellae is illustrated by the results of Moss, Rideal & Bate Smith (29) using monomolecular myosin films. These were more expanded on potassium lactate solutions than on solutions of other salts tried; the expanding action increased with rise in pH. No such effect of ions was found with egg-albumin films. Gorter & van Ormondt (30) found that myosin can be made to spread by adding traces of proteolytic enzyme. They attribute this to the enzymic conversion of the myosin into a substance with spreading properties, but it is possible that breakdown products may be exerting an action similar to that of the lactate ion.

THE STATE OF GLYCOGEN IN THE FIBRES

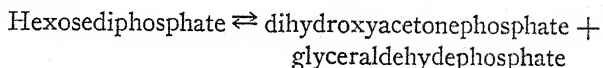
Studnitz (31) has examined histologically the distribution of glycogen in fibres of frog sartorius, using the iodine reaction of Langhans-Romeis. Muscles at rest, at different stages of fatigue, and recovered, were used, the comparisons always being made between left and right muscles of the same frog. The glycogen appeared in the sections in the form of brownish red droplets or granules which seemed to be in the sarcoplasm only. There was often a diffuse coloration, in fibrils as well as sarcoplasm, probably due to the staining of dissolved glycogen or submicroscopic glycogen precipitate. In resting muscle the glycogen was present in all parts of the sarcoplasm, but to a greater extent in the doubly-refracting bands of the fibres. In fatigued muscles, decrease of glycogen in the isotropic bands took place. The longer and more severely the muscle was stimulated, the less glycogen the fibres contained, and the more the portion remaining was concentrated in the anisotropic bands. On recovery, the newly formed glycogen appeared first in the isotropic bands and the original appearance was gradually recovered. Noll (32) and Noll & Becker (33) have used a method which involves the soaking of the muscle for some hours in 3 per cent potassium hydroxide at room temperature. This treatment, by dissolving some protein, renders the glycogen granules more visible after fixing and staining; it probably also renders the desmoglycogen free and visible (34). Noll & Becker compared histological and chemical results on red and white muscle, with a fair degree of agreement, the red muscle showing by

both methods two-thirds to four-fifths of the glycogen content of the white. It is certain that the glycogen does not exist in the living fibre in these granules; they are precipitated by the action of the alcohol. The treatment with potassium hydroxide, though giving a more quantitative picture, probably leads to more displacement of the glycogen than happens if the fibres are put straight into alcohol.

An attempt to investigate the complicated possibilities of glycogen and protein combination in the fibres has been made by Przyłęcki and his collaborators (35, 36, 37, 38). If starch, glycogen or dextrin were added to a myosin solution a precipitate was formed which contained both protein and carbohydrate; with the same materials and conditions the precipitate gave reproducible ratios of myosin to polysaccharide, but the ratio was dependent on a number of factors, such as purity of the myosin, nature and amount of the polysaccharide, pH, and presence of salts. The compounds formed may show considerable stability, being capable of solution and reprecipitation with little or no change in composition. In the case of dextrans, two types of combination were observed: one reversible, from which the dextrin could be removed by repeated washing, the other irreversible. These two types were also found for the glycogen-myosin complex. Any attempts to consider stoichiometric relations in these compounds seem premature as the composition is so variable, according to the conditions, and the molecular weights of the constituents are unknown. Mystkowski (38) extracted rabbit muscle with alkaline potassium chloride solution, and examined for glycogen content the insoluble stroma fraction, the precipitated myosin and globulin-X fractions, and the protein-containing solution. The myosin fraction contained 5 to 23 per cent of the total glycogen and the stroma fraction (consisting also largely of myosin) 12 to 26 per cent. Only 4 to 9 per cent was in the globulin-X fraction, whilst the solution contained 42 to 78 per cent. These results indicate that a large part of the glycogen is bound to protein, especially myosin, although there is little doubt that the extraction of the muscle to obtain the fractions alters the state of combination. Experiments *in vitro* had given no indication that albumins can combine with glycogen, so that it is unlikely that the glycogen in the solution was combined with myogen; probably in the living muscle only a small part of the glycogen is free in solution in the sarcoplasm. Willstätter & Rohdewald (34) give a value of about 65 per cent for desmoglycogen (glycogen bound in such a way that it is not extracted by boiling water or ice-cold trichloroacetic acid).

SOME INTERMEDIATE STAGES IN CARBOHYDRATE BREAKDOWN

Two stages in carbohydrate breakdown by muscle enzymes have received particular attention during the past year¹—triosephosphate production and the phosphorylation of glycogen. Meyerhof, Lohmann & Schuster (39) have shown the presence in dialysed muscle and yeast extracts of an enzyme, aldolase, which brings about the aldol condensation of dihydroxyacetonephosphate with various aldehydes to form ketophosphoric acids. Condensation does not take place between dihydroxyacetonephosphate and other ketones and the suggestion is therefore made that the reaction involved in carbohydrate breakdown is:



The glyceraldehydephosphate undergoes immediate dismutation, or else is converted into dihydroxyacetonephosphate. The condensations described are of considerable intrinsic interest; they have been carried out with formaldehyde, acetaldehyde, glycolaldehyde, propionic aldehyde, lactic aldehyde, methyl glyoxal, glyceric aldehyde, etc.; the reactions appear to be irreversible. No condensations were observed between free trioses or between glyceraldehydephosphate and ketone (dihydroxyacetone).

Earlier work on the coenzyme function of adenylypyrophosphate indicated that a reaction takes place between this compound and glycogen, with formation of hexosediphosphate (40, 41). Parnas and his collaborators have been able to show, however, that glycogen can react in long-dialysed extracts with inorganic phosphate, the product being the Embden hexosemonophosphate (41, 42, 43). If adenylypyrophosphate is added, the Embden ester is further phosphorylated at the expense of the adenylypyrophosphate to hexosediphosphate. Since in muscle extracts poisoned with phlorhizin the formation of the mono-ester from glycogen and free phosphate, or from glycogen and adenylypyrophosphate, is stopped, while the phosphorylation of hexosemonophosphate by adenylypyrophosphate is not inhibited, it is suggested that formation of hexosediphosphate from glycogen goes on in three stages: splitting off of phosphate from adenylypyrophosphate; formation of mono-ester by means of this free phosphate (inhibited by

¹ For the work during 1935 on phosphorylated intermediates in carbohydrate breakdown, as well as on the adenylic compounds and cozymase, see Robison, R., *Ann. Rev. Biochem.*, 5, 181 (1936).

phlorhizin); reaction between the mono-ester and adenylypyrophosphate. Parnas thus suggests that hexosemonophosphate is an intermediate stage in glycolysis and that direct reaction between glycogen and adenylypyrophosphate does not occur; but experiments of Lehmann & Needham (44) seem to show that in long-dialysed extracts with low phosphate content ($0.002 M$), phosphorylation of glycogen goes on at the expense of added adenylypyrophosphate, while at higher phosphate concentrations ($0.01 M$), phosphorylation is primarily from inorganic phosphate even if adenylypyrophosphate is added. When phosphorylation goes on at the expense of adenylypyrophosphate the rate is considerably greater than with inorganic phosphate. Cori & Cori (45), using well-washed minced frog muscle as enzyme preparation incubated in isotonic phosphate, showed that a hexosemonophosphate, apparently the hexose-6-phosphoric acid, is formed at the expense of free phosphate; 4 to 9 mg. of phosphorus per 100 gm. were esterified, but by addition of adenylic acid or adenylypyrophosphate, the amount was greatly increased—to about 50 mg. per cent. If the new ester was added to muscle extract it was converted in a few minutes into the Embden ester. Lehmann & Needham also found the esterification of free phosphate to be increased by adding adenylic acid.

The mechanism of phosphate esterification remains far from clear. There seem to be three possibilities: (a) direct reaction between glycogen and inorganic phosphate in extracts from which all detectable traces of adenylic acid have been removed; (b) transfer of free phosphate to glycogen by means of adenylic acid² in long-dialysed extracts (or well-washed residue) to which no other substrate has been added; (c) esterification of free phosphate, promoted in some obscure manner by simultaneous exothermic reactions, e.g., the reaction between pyruvic acid and triosephosphate. This linking of reactions has been proposed for yeast by Schäffner, Bauer & Berl (46); the same thing is suggested for muscle by the equations given by Meyerhof & Kiessling [47 (p. 86)]. Compare also Dische for erythrocytes (48).

² Parnas [*Ergeb. Enzymforsch.*, 6, 57 (1937)] has found that inosinic acid is almost as effective as adenylic acid in activating esterification of glycogen by free phosphate. As inosinic acid has never been found appreciably active as a phosphate transporter, it is possible that *b* does not involve phosphate transfer. Possibly the same enzyme system is concerned in *a* and *b*, and is capable of activation by adenylic and inosinic acids.

Parnas, Mejbaum & Sobczuk (49) found that phlorhizin inhibits another stage of breakdown—the dismutation of triosephosphate, although it does not stop reaction between pyruvic acid and triosephosphate or glycerolphosphate. They also showed the interesting fact that β -glycerolphosphate, although not a normal intermediate, can react with pyruvic acid providing phosphopyruvic acid more rapidly than does α -glycerolphosphate.

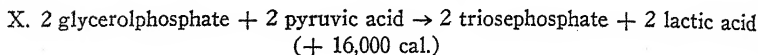
CHEMICAL REACTIONS DURING ANAEROBIC CONTRACTION

The sequence of events in anaerobic contraction may be summarised as follows:

SCHEME I

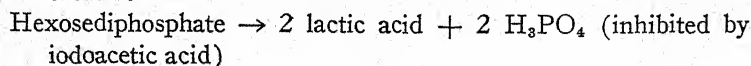
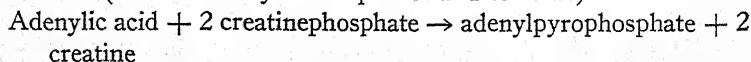
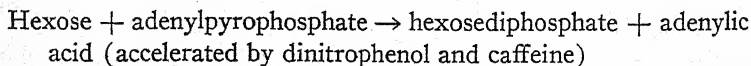
- I. Adenylpyrophosphate \rightarrow adenylic acid + $2H_3PO_4$ (+24,000 cal.)
- II. 2 adenylpyrophosphate + glycogen \rightarrow 2 adenylic acid + 2 hexosediphosphate (+24,000 cal.)
- III. 2 creatinephosphate + adenylic acid \rightarrow adenylpyrophosphate + 2 creatine (\approx 1000 cal.)
- IV. 2 hexosediphosphate \rightleftharpoons 2 dihydroxyacetonephosphate + 2 glyceraldehyde-phosphate ($-28,000$ cal. if \rightarrow ; $+28,000$ cal. if \leftarrow)
- V. 4 triosephosphate \rightarrow 2 3-phosphoglyceric acid + 2 glycerolphosphate (+28,000 cal.)
- VI. 2 3-phosphoglyceric acid \rightleftharpoons 2 2-phosphoglyceric acid (+0 cal.)
- VII. 2 2-phosphoglyceric acid \rightleftharpoons 2 phosphopyruvic acid (+0 cal.)
- VIII. 2 phosphopyruvic acid + adenylic acid \rightarrow 2 pyruvic acid + adenylpyrophosphate ($-7,400$ cal.)
- II. adenylpyrophosphate + glycogen \rightarrow hexosediphosphate + adenylic acid
- IV. hexosediphosphate \rightarrow 2 triosephosphate
- IX. 2 triosephosphate + 2 pyruvic acid \rightarrow 2 phosphoglyceric acid + 2 lactic acid (+16,000 cal.)
- VII. 2 phosphoglyceric acid \rightarrow 2 phosphopyruvic acid

By further reaction between the phosphopyruvic and adenylic acids, more pyruvic acid is formed which reacts with more triosephosphate (formed by further phosphorylation of glycogen by means of the adenylpyrophosphate). The contraction stimulus presumably initiates reactions I or II or both and the other reactions (III to IX) follow on these. When the response is over, and reactions I to IX have ceased, an excess of equivalent amounts of glycerolphosphate and pyruvic acid remains, which then react together according to reaction X.



The triosephosphate thus formed and equivalent adenylic acid eventually give lactic acid and adenylypyrophosphate. Meyerhof & Kiessling (47) have shown that reaction IX is much more rapid than reaction X and the same thing has been found by Parnas, Sobczuk & Mejbaum by the method of suppression of ammonia formation (50). [For a forecast of the possibility of reaction IX see Parnas (50a).] It seems reasonable to suppose, therefore, that, as long as triosephosphate is being formed, the pyruvic acid will react with this, leaving the small concentration of glycerolphosphate formed initially to be broken down in the final stages. It is, of course, possible that resting muscle normally contains traces of pyruvic acid, which would react directly with the triosephosphate as soon as it is formed; in this case, glycerolphosphate formation would not occur in normal contraction.

During an anaërobic contraction inorganic phosphate is set free; as the only known reaction in muscle leading to formation of free phosphate is reaction I, this is introduced into the scheme. Under a variety of conditions the phosphate set free is equivalent to the phosphagen broken down [see Lundsgaard (51)]; the above scheme gives no explanation of this correlation, for there is no reason apparent why the extent to which I takes place should depend upon the extent to which III takes place. Cori & Cori (52, 53) have shown that in intact muscle, soaked in a solution containing dinitrophenol or caffeine, the markedly increased lactic acid production is accompanied by a fall in creatinephosphate with an equivalent increase in inorganic phosphate. If iodoacetate is added, as well as one of these poisons, the creatinephosphate phosphorus appears as hexosediphosphate phosphorus. These facts lead Cori & Cori to the conclusion that the inorganic phosphorus formed in the first cases had passed through the hexose ester stage, and apparently to the postulation of some phosphatase action (other than that of adenylypyrophosphatase) not yet directly demonstrated.



During anaërobic contraction some of the creatinephosphate phosphorus may appear as hexosemonophosphate phosphorus instead of inorganic phosphorus [see, e.g., Fisher & Cori (54)]. This accumulation of hexosemonophosphate presumably goes on by means of one of the mechanisms already considered for esterification of inorganic phosphorus, and is believed by Cori & Cori to be a reaction independent of lactic acid formation (53). Exactly at what stage glycogenolysis begins, and when phosphorylation of adenylic acid is taken over by phosphopyruvic acid rather than creatinephosphate, is not known; but even in the smallest tension productions which can be examined chemically, some lactic acid formation goes on during the contraction period [Lundsgaard (51)].

During anaërobic recovery about one-third of the lost creatinephosphate is resynthesized; there is an equivalent esterification of inorganic phosphate, and further lactic acid formation. As an example of the many possible mechanisms which might be postulated for the anaërobic recovery phase we may write:

SCHEME II

- I. Glycogen + $2H_3PO_4 \rightarrow 2$ hexosemonophosphate (+0 cal.)
- II. 2 hexosemonophosphate + adenylypyrophosphate $\rightarrow 2$ hexosediphosphate + adenylic acid (+24,000 cal.)
- III. 2 hexosediphosphate $\rightleftharpoons 4$ triosephosphate (−28,000 cal. if \rightarrow ; +28,000 cal. if \leftarrow)
- IV. 4 triosephosphate + 4 pyruvic acid $\rightarrow 4$ phosphoglyceric acid + 4 lactic acid (+32,000 cal.)
- V. 4 phosphoglyceric acid $\rightarrow 4$ phosphopyruvic acid (+0 cal.)
- VI. 2 phosphopyruvic + adenylic acid $\rightarrow 2$ pyruvic + adenylypyrophosphate (−7,400 cal.)
- VII. adenylypyrophosphate + 2 creatine $\rightarrow 2$ creatinephosphate + adenylic acid (+ *circ.* 1000 cal.)
- VIII. 2 phosphopyruvic acid + adenylic acid $\rightarrow 2$ pyruvic + adenylypyrophosphate (−7,400 cal.)

In this scheme an excess of phosphopyruvic acid is provided at stage V, above what is needed to re-phosphorylate the adenylic acid; it is suggested that the excess passes via adenylic acid to creatine. The scheme gives no clue as to why resynthesis of creatinephosphate should stop when only one-third finished.

The data given in Schemes I and II for heats of reaction are obtained from the papers of Lohmann, Meyerhof & Schulz (55, 56, 57, 58). It will be observed in Scheme I that little heat is taken in or

given out as a result of the sum of the glycogenolytic reactions as far as the formation of phosphopyruvic acid. The hydrolysis of phosphopyruvic acid sets free 8,300 cal. per mol. The heat set free when one mol of lactic acid is formed from glycogen is 16,200 cal., so that just over half is liberated at the pyruvic acid stage. The rest is set free when the pyruvic acid reacts with glycerolphosphate; here a production of 8,000 cal. was observed per mol of lactic acid formed and presumably the same heat relations hold when pyruvic acid reacts with triosephosphate although these have not yet been measured. There is, further, the heat produced when the acid formed is neutralised by protein—of the order of 6,000 cal. per mol of lactic acid. The reversible reaction of Lohmann goes on with very small heat exchange, but it is interesting that the heat obtained from breakdown of two molecules of phosphopyruvic acid is not enough to cover the requirements of the endothermic resynthesis of one molecule of adenylypyrophosphate. During resynthesis of creatinephosphate in muscle extracts at the expense of phosphopyruvic acid phosphorus a negative heat of 3,200 cal. per mol was indeed observed (57). It is very probable that this reaction underlies the negative heat observed by Hartree (59) after the initial heat of anaërobic contraction. Here for a brief space the endothermic reaction outstrips the accompanying exothermic reactions. The figures of Lundsgaard show a resynthesis of about two molecules of phosphagen for each molecule of lactic acid formed. This relation does not agree with the above equations, nor with any based solely on the Parnas reaction. Further work is needed. Both Meyerhof & Schulz (57) and Murali (60) have discussed the unsatisfactory but inevitable expedient of using heats of reaction instead of changes in free energy in these calculations.

Lehmann & Needham (44) have examined the relative rates of various phosphorylations in muscle extract, and have attempted to correlate the results with happenings *in vivo*. The reaction between creatine and adenylypyrophosphate is always much more rapid than the reaction between glycogen and adenylypyrophosphate. In alkaline solution adenylic acid reacts more rapidly with phosphopyruvic acid than with creatinephosphate; in acid the reverse is true.

SOME COENZYME SYSTEMS CONCERNED IN GLYCOLYSIS

The part played by adenylypyrophosphate in phosphorylation of glycogen has already been discussed. The coenzyme function of adenylic acid in dephosphorylation of phosphopyruvic acid was eluci-

dated in 1935 by several independent groups of workers on muscle extract (61, 62, 63, 64) who demonstrated also the transfer of phosphate to creatine (reverse Lohmann reaction). This work followed on the pioneer experiments of the Parnas school on muscle brei.

Lehmann (65) showed that the reaction between adenylypyrophosphate and creatine or arginine is made up of two reversible bimolecular reactions

- (1) adenylypyrophosphate + creatine \rightleftharpoons adenosinediphosphate +
creatinephosphate
- (2) adenosinediphosphate + creatine \rightleftharpoons adenylic acid +
creatinephosphate

He determined the equilibrium constants, and found K for reaction (1) greater than for reaction (2).

That cozymase can function as a transporter of phosphate in muscle extract was shown by Euler & Vestin and Vestin (66, 67); their results, as well as those of Needham (68), indicate that a cozymasepyrophosphate is formed.³ Meyerhof & Kiessling (69) were able to prepare the acid sodium salt of this compound from yeast extract to which cozymase and phosphopyruvic acid had been added.

The part played by the nicotinic acid amide group of cozymase in the reaction between acetaldehyde and phosphorylated sugar has been conclusively demonstrated by Warburg & Christian (70). That cozymase should be essential also in muscle glycolysis at the stage of reaction between pyruvic acid and triosephosphate was to be expected. Meyerhof & Ohlmeier (71) have shown that this is the case. In the case of muscle extracts dialysed thirty-six to forty-eight hours, lactic acid formation from the reaction of pyruvic acid with hexosediphosphate could not be re-initiated by adding adenylic acid, but could be restored by adding cozymase (1 to 5 μ g. per cc.) to the extent of 50 per cent of the maximal. By addition of 180 μ g. of adenylic acid as well, or of 150 μ g. of cozymase, the lactic acid formation could be raised to maximal. Here the cozymase seems to be functioning as both hydrogen transporter and phosphate transporter, although the exact sequence of reactions is not yet clear. Such a low concentration

³ In later papers [Schlenk, F., & Euler, H. v., *Naturwissenschaften*, 24, 794 (1936); Euler, H. v., & Alder, E., *Z. physiol. Chem.*, 246, 83 (1937)] it is stated that very highly purified cozymase can act as phosphate transporter only in certain circumstances. The results are very complicated and the mechanism of the reactions involved is not yet understood.

suffices for the former function that only in extracts dialysed for a very long time can the effect of its absence and of its addition be seen. It is possible that the reactivating effect of *Kochsaft* described by Kendal & Stickland on starch glycolysis in highly diluted muscle extract is due to its cozymase content (72).

CONTRACTION AND RECOVERY UNDER AËROBIC CONDITIONS

For frog's muscle Gemmill (73) has shown that other material than carbohydrate must be used to supply the energy (to the extent of 58 per cent) in aërobic contraction, even when the conditions are not severe. The R.Q. for resting frog muscle he found to be 0.80, for stimulated muscle 0.90 (74). When hexosemonophosphate has accumulated in muscle, as the result of action of epinephrine or of stimulation, during oxidative recovery of the isolated muscle (frog) some of this ester is converted into glycogen (75); very little is oxidised. The same results were obtained with muscles poisoned with iodoacetic acid. Sacks & Sacks (76) confirm the results of many previous workers in finding that no glycogen synthesis from lactic acid takes place during recovery in mammalian muscle. They used rabbit gastrocnemii with circulation and nerve supply intact.

The suggestion has often been made that resynthesis of creatine-phosphate may go on during aërobic contraction at the expense of the oxidation of various substances, including fat, and that the formation of lactic acid is not a necessary accompaniment. This view is supported by experiments of Grimlund (77) which show that in frog sartorius and heart, poisoned with bromoacetic acid, the oxygen uptake and the capability of work in oxygen are increased by adding lactate, pyruvate, succinate, fumarate, or phosphoglycerate, provided that these substances can enter the tissue. Evidence continues to accumulate that in the whole organism muscular activity can go on without lactic acid formation if the conditions are sufficiently aërobic. Bang (78) measured the blood lactate during and after exercise in man. A rapid initial rise was followed by a steady decrease in blood lactate during the exercise, and this fall was not accelerated by cessation of the exercise. The interpretation made is that conditions are first anaërobic and lactic acid is formed; but after adjustment of the respiration and circulation lactic acid formation ceases, oxidations taking on the burden of phosphagen resynthesis; the accumulated lactic acid in the muscle diffuses out, and this process is unaffected whether the work continues or not.

The investigations of Millikan (79) raise the question as to whether oxidations may participate even more directly in the energy supply, at any rate in red mammalian muscle with normal circulation. By means of the differential photo-cell colorimeter he was able to measure the changes in oxygen saturation of muscle hemoglobin during rest or contraction; the thin muscle used, the cat soleus, could be arranged with the minimum of dissection and no disturbance of blood or nerve supply, using a special trough for illumination. The results, with three seconds to ten seconds of tetanus, show that contraction is accompanied by an immediate change of some $O_2\text{MHb}$ (oxymuscle-hemoglobin) into MHb , the degree of change depending on the length of the stimulus. The response time of the instrument is only 0.2 sec., so that within these limits the beginning of oxygen utilisation is simultaneous with the onset of contraction. This is the first time that technique has been available for investigating the time relations of oxygen uptake and beginning of contraction and the results are of great interest [see also Kramer (80)]. Cessation of the contraction leads at once to a rise in the $O_2\text{MHb}$, which within a few seconds returns to normal. Whether the oxidations concerned supply energy for creatinephosphate resynthesis, for adenylypyrophosphate resynthesis, or for some still earlier stage we can only guess. There seems no doubt that oxidation of lactic acid is too slow a process to be concerned, and the data available for aërobic phosphagen resynthesis suggest that this also is too slow [Sacks & Sacks (81); Muralt (24)].

As regards the rapid recovery of the $O_2\text{MHb}$, it must be remembered that this does not necessarily mean that all excess metabolites have already been removed; for the reaction of MHb with oxygen, when available, is so rapid that it becomes itself reoxidised as rapidly as it passes on oxygen. The oxygen consumption, calculated from disappearance of $O_2\text{MHb}$, is of the same order as the total increased oxygen consumption found by the usual metabolic methods, but the reliability of the latter value is not enough to tell us whether the oxygen consumption calculated here is 100 per cent or some fraction of the total. The chemical results of Sacks & Sacks (82) were obtained on rabbit and rat gastrocnemius using conditions apparently similar to those of Millikan—nerve and blood supply intact. After five seconds of tetanus an accumulation of 38 mg. per cent of lactic acid was found, and a phosphagen breakdown corresponding to 14 mg. of phosphorus per 100 gm. of tissue. Here an oxygen debt within the muscle seems unavoidable. There was relatively less lactic acid for-

mation with longer stimuli, i.e., the conditions became more aërobic on account of improved circulation.

The attempt may be made to correlate Millikan's results with the observations on recovery heat in aërobic contraction. In the only recorded experiments on red mammalian muscle [isolated dog scale-nus, Cattell & Shorr (83)], the recovery heat is about three times the initial heat; these experiments were made, however, at 20°, and at 37° the recovery heat would have been pushed much further toward, and probably into, the contraction period. It has been observed that the proportion of the whole heat found in the recovery phase diminishes as the amount of contraction diminishes [Muralt (60)], being less for a twitch than for a short tetanus. Bugnard (84) has suggested that the lower the degree of activity and the less the oxygen want, the more directly the energy of oxidation is used.

Both Hill (85) and Millikan (86) have demonstrated the special suitability of muscle hemoglobin, because its oxygen affinity is higher than that of blood hemoglobin, to act as an oxygen store, tiding the muscle over times of intermittent oxygen supply or consumption. A method of estimating muscle hemoglobin in presence of blood is described by Watson (86a).

COMPARATIVE WORK

Lohmann (87) has shown that the phosphagen of *Octopus* is argininephosphate, and has isolated this compound from *Octopus* muscle. Lehmann (64) found that vertebrate muscle will not phosphorylate added arginine, nor will crab muscle phosphorylate creatine. Baldwin & Needham (88), therefore, regard their finding that extracts of echinoid muscle can esterify both creatine and arginine as evidence confirming the presence of both phosphagens in these muscles. Further study of the Echinodermata showed that the muscle of *Holothuria tubulosa* can synthesize argininephosphate but not creatinephosphate, and contains only argininephosphate. The Crinoidea also contain only argininephosphate, the Ophiuroidea, on the other hand, only creatinephosphate. The transfer of phosphate takes place in all these invertebrate muscles by a mechanism very similar to that in vertebrate muscle. The chief difference observed was that in *Octopus*, as in crab muscle, whilst phosphorylation of adenosine-diphosphate readily takes place, phosphorylation of adenylic acid seems to be very slow; the echinoderm muscles which were studied resembled vertebrate muscle in their greater capability of phosphory-

lating adenylic acid. Lohmann (89) found that crab and *Octopus* muscle deaminate adenylic acid much more slowly than vertebrate muscle.

Verjbinckaya, Borsuk & Kreps (90) report the presence of two phosphagens, presumably the arginine and creatine compounds, in the holothurian *Cucumaria frondosa*, but it has not been possible to consult the original paper. Cockroach leg muscles contain arginine-phosphate (91).

Carnosine and anserine have been found together in the skeletal muscles of a number of mammals: dog, cat, deer, gnu, and opossum (92). In the dog and gnu anserine was certainly in excess, possibly also in the others. Previously the two substances had been found together only in the crocodile (93). The random distribution of these substances in the animal kingdom becomes always more pronounced. No hint as to their function in skeletal muscle has so far been obtained. Sawronj (94) found always more carnosine in white muscle than in red.

THE ACTION OF HORMONES, VITAMINS, ETC., ON CHEMICAL CHANGES IN MUSCLE

Nachmansohn and his colleagues (95, 96), extending earlier work of Hegnauer & Cori (97), found that when excised frog muscles were soaked in Ringer solution containing adrenaline, the resting anaërobic lactic acid production was increased to 20 to 50 per cent above normal; the creatinephosphate content remained constant for some hours, then showed a definite rise. The effects of parasympathomimetic substances on these reactions were also studied (98, 99, 100, 101, 102). Acetylcholine (200 µg. per cc.), pilocarpine (0.3 per cent), and potassium (five to eight times the normal concentration in Ringer) all caused increased lactic acid formation, accompanied, however, by increased breakdown of creatinephosphate. This last distinction would seem to need confirmation as Tipton (103) has found creatinephosphate synthesis accompanying increased lactic acid formation anaërobically, and increased oxygen uptake aërobically, in potassium-poisoned muscles. Increased heat production in oxygen (up to ten times normal) was observed with similar treatment [Solandt (104)]. This effect could be reversed by washing with normal Ringer solution. It was not necessarily connected with loss of excitability, but the increased oxygen uptake found by Fenn & Cobb

(105) for muscles made inexcitable by potassium may also be mentioned here.

Grégoire (106) found the curious result that tissues of animals injected with thyroxine have a greater capacity for lactic acid formation than normal tissues although the rate of glycolysis is not increased. This effect was not further analysed.

As regards deprivation of adrenal hormones, Silvette & Britton (107) have found decreased muscle glycogen in adrenalectomised marmots and opossums. These results are of interest because the glycogen decrease was accompanied by a rise, not a fall, in the sodium and chloride content of muscle and serum, and they throw emphasis on deranged carbohydrate metabolism as the main deleterious result of adrenalectomy. Moschini (108) confirmed the fall in the creatine-phosphate content of muscles of adrenalectomised frogs. Muscles of adrenalectomised frogs show a slightly diminished pH (6.90 to 7.35 instead of the normal 7.2 to 7.4) and a decreased buffering power (to one-half or three-fifths of the normal). The muscle proteins show considerably less solubility than normal in three times isotonic phosphate solution at pH 7.5 [Benetato (109)].

Grégoire (110) found in pigeons suffering from avitaminosis B an increase in the magnesium content of muscle from 25 mg. (normal) to 35 mg. per 100 gm. of muscle; this effect was not seen in inanition, where the magnesium content falls. In both states there is a gradual fall in adenylypyrophosphate. Murza-Murzicz & Bohdanowiczówna (111) independently observed the magnesium rise.

PRODUCTION OF A HORMONE DURING MUSCLE CONTRACTION

Muscle contraction (dog) was accompanied by a large increase in histamine content of the venous blood. It was calculated that the gastrocnemius, weighing 42 gm., produced 19 μ g. during maximal tetanic contraction [Anrep & Barsoum (112)].

PRODUCTION OF ACETYLCHOLINE AT MOTOR NERVE ENDINGS IN VOLUNTARY MUSCLE

Dale and his collaborators have obtained incontrovertible evidence that acetylcholine is liberated when voluntary muscle is stimulated through the motor nerve (113, 114). This result was obtained after complete removal of all autonomic and sensory innervation of the muscles in question (cat's tongue and gastrocnemius, dog's quadriceps extensor femoris, frog's hind legs). The perfusion fluid was

Locke or Ringer solution containing one part of eserine in five million. The highest concentrations of acetylcholine were observed with the cat's tongue where the content of the effluent during stimulation was 1 in 5×10^7 . The amount actually collected in one minute, apart from what escaped by anastomotic channels, was 0.06 μ g. Direct stimulation of the muscle caused acetylcholine formation if the nerve supply was normal; after degeneration of the nerves, contraction obtained by direct stimulation was accompanied by no trace of acetylcholine formation. After application of curare, which prevented the transmission of impulse from nerve to muscle, stimulation of the nerve still led to acetylcholine production. When, owing to exhaustion, stimulation of the nerve no longer called forth a contraction, no acetylcholine appeared as a result of the stimulation.

Brown, Dale & Feldberg (115) further showed for mammalian muscle that acetylcholine, if injected rapidly enough directly into the empty arteries of a normal muscle, caused contraction of the muscle at not less than half the speed of a maximal motor nerve twitch. Injection of 2 μ g. caused a contraction with a tension similar to that of such a twitch. This response was abolished by curare. The response to acetylcholine injection, though superficially resembling a twitch, was actually a short, asynchronous tetanus. This effect is to be explained by the persistence after injection of a supraliminal concentration of acetylcholine near the endplates, causing by diffusion a repeated response. The explanation requires the assumption that acetylcholine disappears more rapidly from the neighbourhood of the nerve endings than from the general body of the muscle, i.e., that there is a local concentration of cholinesterase in the muscle near the endings. The action of eserine, which causes the response to a single nerve volley to be repetitive, not single, bears out this explanation.

The exact site and the chemical mechanism of the liberation of acetylcholine are unknown. Lopicque (116) suggests that the formation takes place over the whole endplate, i.e., in the muscle itself. In this way its effect is to provide a new stimulus, and one which is more widely extended spatially, which is probably of importance.

RESPIRATION

The advances in knowledge of oxidation mechanism are of general interest and concern all tissues. We may, however, mention, because the experimental work has been so largely on muscle tissue, the important conception of Szent-Györgyi and his coworkers (117, 118),

who regard the fumaric-oxalacetic system with its dehydrogenase as a transport mechanism conveying activated hydrogen from metabolites to the cytochrome-indophenoloxidase system. Although Innes (119) has shown that, in the concentrations considered necessary by Szent-Györgyi, fumaric acid is acting as substrate, Stare & Baumann (120) have found that very much smaller concentrations of fumaric acid activate larger oxygen uptakes than can be accounted for by their own oxidation.

Fenn (121) has made the observation that the anaërobic carbon dioxide, liberated from frog's heart after a period in nitrogen, is greater than the corrected preformed carbon dioxide. The suggestion that dehydrogenations involving carbon dioxide formation have occurred at the expense of other substances than oxygen is interesting, particularly in view of the work of Krebs (122).

MUSCULAR DYSTROPHY

Morgulis & Spencer (123) have studied the changes in metabolism, and in the constitution of the blood and various organs, during development of nutritional muscular dystrophy in rabbits, and during recovery from the disease. The glycogen content and all the acid-soluble phosphorus fractions were reduced in the skeletal muscle of the dystrophic animals, while the cholesterol content was greatly increased. Prevention or cure of muscular dystrophy could be effected by supplementing the dystrophic diet with fresh green alfalfa, lettuce and vitamin E, dry alfalfa and vitamin E, or whole wheat germ.

Milhorat & Toscani found that ingestion of glycollic acid by patients suffering from progressive muscular dystrophy led to a small increase in creatine output, but the effect was much less than that obtained by feeding glycine (124). The presence of simple non-methylated guanidine derivatives in the urine of such patients has been demonstrated by Sullivan, Hess & Irreverre (125).

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THE METABOLISM OF BRAIN AND NERVE*

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Major trends in the chemical investigation of the nervous system begun or flowering in the past two years include the study of the following: metabolic concomitants of disease, of vitamin and hormone disturbances, and of the action of narcotics and other drugs; glycolytic enzymes and sequences, and especially the rôle of phosphates; metabolism of amines; neurohumors; and histochemistry. Renewed attention has been directed to salt relations and respiratory mechanisms have been further explored and analyzed. Since biochemical workers, not specifically concerned with the nervous system, are recognizing more and more the usefulness of brain as a source of active preparations, much incidental information has accumulated regarding it. Also, the burst of knowledge concerning the physical behavior of neural structures, especially as regards electrical excitation on the one hand and electrical manifestations of activity on the other, is being more and more integrated with chemical data.

Vitamin C.—The "non-carbohydrate" reducing substance of brain turns out to be largely ascorbic acid, identified by oxidation with 2,6-dichlorophenolindophenol (1, 2), by spectroscopic methods (1), by its vitamin-C action (3, 4), and by isolation (3). In brain it may exist in combined form (3, 5). Quantitative values obtained by various workers (e.g., 1, 6, 7) are in satisfactory agreement. The cerebellum is richest (25 mg. per cent, in the human); cerebrum and brain stem contains less (12 to 17 mg. per cent), and sciatic nerve is distinctly poor (3 mg. per cent), even compared to muscle. The concentration decreases, from species to species, with increasing size, and in individuals with advancing age; a rôle in growth has also been urged (8). It is interesting that, as regards age and species, reducing substance concentration parallels the rate of oxygen consumption, indicating participation in oxidations; but the relation breaks down in that the toad has a higher concentration than the rat (4) and that grey and white matter have equal vitamin-C contents (1, 8; but see 4) and quite different respiration rates. The point is worth further study since, in general, respiration intensity does parallel vitamin-C con-

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tent (9). Incidentally, the pars intermedia of the pituitary is 50 per cent richer in vitamin C than even the adrenal cortex (10).

Diet markedly affects the ascorbic acid content of brain; simple starvation may reduce it (11), but a scorbutic diet is much more effective in guinea pigs (12, 13) although not in rats or rabbits (7). Oxidized or reduced (dihydro) ascorbic acid added to a scorbutic diet relieves symptoms and increases the brain vitamin-C content toward normal (12). A high content of vitamin C in cerebrospinal fluid is reported in hypertension in humans, a strikingly low one in multiple sclerosis (2), and no change in stupor, sleep, or narcosis (8). The reported synthesis of ascorbic acid from mannose, *in vitro*, is not confirmed in experiments with rat brain or liver (14). A third of the total brain glutathione (78 mg. per cent) is in the oxidized state (15), of interest relative to the redox relation between this substance and ascorbic acid (16, 17).

Vitamin A.—Vitamin A is essential to the normal metabolism of nervous tissue since distinct lesions appear in the brain and peripheral nerves of animals on A-deficient diets (18). The carotene content of frog nerve falls markedly on starvation, while carotene plus xanthophyll, when fed, are converted into vitamin A (19). Demyelination and desaturation of fats (20) after a week or two of vitamin-A deficiency suggest a markedly disturbed lipid metabolism; and indeed much evidence now indicates that myelin is chemically very labile and depends on a delicate metabolic balance for maintained integrity (21). Its rapid loss with disturbed oxidations, as in fiber degeneration, venous stasis (22), decreased oxygen supply, or certain diseases (as dementia praecox), or with toxin accumulation, comes to mind. Further, particular lipoids, largely or solely in the ganglion cells, are involved in disease, as indicated by the increase in sphingomyelin and the decrease in unsaturated fatty acids in amaurotic family idiocy (23), or by the cerebroside increase in Gaucher's disease. Pathologic variations in characteristic lipid inclusions of brain cells have also been described (24), and parenteral administration of lipocerebrin (25) but not of cholesterol (26) increase brain lipoids. It is relevant that vitamin A is a precursor of visual purple in retinal function and that a unique carotene is formed from it under the influence of light (27). Finally, vitamin E is also essential to neural integrity, its chronic lack leading to symptoms much like those of vitamin-A deficiency (28).

Vitamin B₁.—Vitamin-B₁ deficiency evokes central lesions before

peripheral nerve or receptors are affected (29); these lesions center about the vestibular nuclei (29, 30). Disturbed function (nystagmus, etc.) accompanies metabolic defects and precedes anatomic degeneration; hence the missing factor can restore practically normal function in twelve to twenty-four hours, beginning within two. The metabolic errors are by no means limited to the nervous system but may be even more striking in the heart or kidney (31).

The chemical lesion of beri-beri, in the rat as in the pigeon, leads to accumulation of pyruvate (bisulphite-binding substance) in macerated brain in Ringer-lactate-pyrophosphate solution, and some decrease in oxygen consumption is also observed; addition of the vitamin *in vitro* restores these toward normal (catatorulin effect, 32). The cerebellum is perhaps most affected. The respiratory defects are already present in the brains of pigeons in an early (leg weakness) stage of beri-beri (33). Fluoride and iodoacetate alike depress the respiration of avitaminous brain and decrease or abolish the catatorulin effect (34). Dichlorodiethylsulphone acts similarly in far smaller concentrations (35) and, like iodoacetate, causes accumulation of pyruvate, while fluoride suppresses pyruvate production. Glutathione, added beforehand, can destroy the sulphone and iodoacetate and their actions, but it cannot reverse the effects when established. Vitamin B₁, although not inactivated, is unable to counteract these toxic effects. For the sequence of lactate oxidation (lactate \rightarrow pyruvate \rightarrow carbon dioxide), fluoride inhibits the first step, the sulphone and iodoacetate the second. Vitamin B₁ is also concerned, mainly, with the second step since in its absence pyruvate accumulates. Further, a lack of vitamin B₁ interferes less with lactate than with pyruvate oxidation (31), and this interference may be secondary to pyruvate accumulation, which does depress lactate oxidation. Complete loss of the ability of lactate to hasten the reduction of methylene blue has been reported (36), however, in the avitaminous chicken medulla. An additional complication appears with the temperature at which brain is minced, for in the cold one-third of the lactate respiration is irreversibly lost (34), possibly by oxidation of the lactic coenzyme, and the catatorulin effect is diminished. The Oxford workers postulate the reaction of an unknown substance with vitamin B₁ to give the agent which actively enters into the lactate and pyruvate oxidations. Even with an adequate supply of vitamin B₁, oxidation is not normal, and more pyruvate disappears than can be fully oxidized (31). Succinate oxidation is not affected by the temperature

of preparation (34) nor by vitamin B₁ (36). In most of these experiments lactate was the only substrate, and pyrophosphate, which lessens the fall of respiration with time and increases the catatorulin effect (32, 34), was present. The implications of these data for normal brain oxidations with carbohydrate as substrate will be considered later. A reported increase in potassium in beri-beri brain is not confirmed (37).

Hormones.—Thyroxin increases the respiration of nerve and brain (38). By comparing the inhibitory effects of cyanide, narcotics, etc., on the respiratory rate (Q_{O_2}) of brain in the presence of various substrates, the conclusion has been drawn (38, 62, p. 194) that this hormone increases by several times the cell content of those dehydrogenases involved in the oxidation of carbohydrates and their intermediates. The hemin oxidase-cytochrome system is also increased, but to a lesser extent. Similar conclusions have been reached by analysis of the synergism between thyroxin and dinitrophenol (39). (The phenol, presumably serving as a hydrogen carrier, can manifest a greater effect when an excess of dehydrogenase makes hydrogen transport the slowest step in the oxidation sequence.) It is claimed that the thyroid hormone must enter cells along their innervating nerve fibers to be effective (40).

Cortin influences the potassium and sodium balance in the brain and cerebrospinal fluid, as elsewhere (see later). It also enhances conditioned reflexes in sheep and relieves "neuroses" produced by situations involving too difficult discrimination (41). Adrenaline and acetylcholine have opposite effects, and since the action of these agents is closely bound to cations, there may be a relation between the improved cerebral function and cerebral salt content. Adrenaline also depresses the knee jerk (42), although possibly by way of a central vasoconstriction. A rôle similar to that of cortin is suggested for testicular hormones, since castration disturbs conditioned reflexes and predisposes to neuroses in dogs (45).

A postulated relation of bromine (contained in a pituitary hormone) to mental disease has not been supported (43), nor is the bromine content of brain high in relation to other organs. On administration, bromide accumulates most in the medulla and least in the cerebrum, being bound to lipoids rather than to proteins (44). Specific bromide effects on conditioned reflexes (45), and on the after-discharge in voluntary contraction (46) have been reported.

Narcotics.—Narcotic action in relation to respiration has received

much attention. Although it is accepted that narcotics can depress dehydrogenases, there is disagreement as to whether the doses used in producing ordinary narcosis can do so (see 47). Several new observations speak against this occurring *in vivo*. The more rapid falling off of the oxygen consumption of brain removed from anesthetized rats than of that from controls is attributed to the diminished carbohydrate content of the former (48); for ether, ethylene, and alcohol evoke an adrenaline discharge and effect a mobilization of tissue sugar, including that of brain, to produce hyperglycemia. Adrenaline injection does affect the respiration of subsequently isolated brain in the same way as ether; glucose added *in vitro*, by restoring substrate, re-establishes the normal oxygen consumption curve for brain from anesthetized animals; and amytal, which antagonizes the glycogenolytic action of ether, also prevents the rapid fall of respiration of the brain *in vitro*. Fasting and insulin also lower respiration (49). Alcohol, serving as fuel, can increase oxygen usage a third (49) and excessive morphine has been reported to have a similar action (50). Additional data on barbiturates, local anesthetics, and alkaloids are available (49). Chloral, morphine, and barbiturates, incidentally, greatly diminish the normal hyperglycemic curve following glucose administration (51).

In the presence of adequate substrate (lactate, succinate), or during the short times required for methylene blue reduction, it is reported (52) that the oxidation rate of brain tissue from animals anesthetized with ether, chloroform, avertin, or magnesium is not decreased. In fact, the time for reduction of methylene blue may be decreased a third for brains of anesthetized chickens or guinea pigs, or of animals similarly immobilized by "hypnosis" before decapitation, as compared to normals. Changes in brain carbohydrate or lactate are controlled and a direct influence on dehydrogenases excluded. The authors attribute these striking and persistent metabolic changes to simple immobilization, findings and conclusions that merit examination by others.

Determination of narcotic concentration in varying brain regions after systemic administration, has hardly supported the idea of specific affinities (53) although the duration of narcosis is related to the rate of disappearance of the drug (54). There is ample evidence that brain is not a homogeneous tissue metabolically any more than in its anatomical or chemical structure or its function, so that various chemicals might well have different actions on specific regions. Evi-

dence for this is seen in the local lesions with vitamin lack, for example, and other relevant facts have been summarized elsewhere (62, p. 194). The view that barbiturates block conduction in the thalamus, while ether, chloroform, etc., depress the cortex (55), although supported by some electrical studies (55, 56) is rendered doubtful by others (57, 58). The respiration of small tissue plugs from various brain regions has not shown differential depression with ether or nembutal, in preliminary experiments (59). The time course of the respiratory inhibition produced by these drugs (luminal, evipan, ether) is differently affected by potassium, and shows different temperature coefficients (60). Oxidation of glucose is most inhibited in brain, that of pyruvate in liver and kidney.

Oxygen requirement.—The blood supply to the brain and the control of this supply have been fully reviewed recently (61), and the influence of oxygen-lack and changes in carbon dioxide concentration on brain potentials have been considered by several participants in a symposium on neural activity (62, pp. 285, 292, 320). Humans subjected to insufficient oxygen supply show defective cortical function, as shown by writing (63) and word-association tests (64), psychic disturbances (65), diminished visual after-images and intensity discrimination, lowered auditory sensitivity, and the like (66). More severe gaseous unbalance is required to disturb the function of the brain stem (rotational nystagmus as the measure) than of the cortex (66). Severe reduction of the cerebral blood supply in dogs leads similarly to marked, often permanent, interference with finer discriminations as measured by conditioned reflexes (67). Excessive oxygen or nitrogen pressures (oxygen or air at 3 atmospheres) also affect the brain particularly: humans show inattention, syncope and convulsions in oxygen, and euphoria merging into drowsiness in air (68).

Especially interesting are experiments in which the circulation of dogs is completely stopped and, after varying intervals, re-initiated (69). After thirty or even sixty minutes of anoxia, neurones in the brain stem can regain function (respiration, vasomotor and pupillary reflexes). After only ten to fifteen minutes, additional midbrain cells recover and produce decerebrate rigidity. Oxygen-lack longer than five minutes destroys cerebral function and causes delayed death, but up until this time full recovery (except for blindness) can be attained. Neurones can, then, remain functionally blocked by anoxia for some time before the damage is irreparable. The times required

for temporary and for irreversible loss of function are probably determined by the metabolic rates of the various cells, those with greater respiration suffering earliest—as in peripheral nerve. [But see evidence that, in man, local ischemia first lowers the nerve thresholds and then blocks the larger fibers before the small (70).]

Respiration at rest.—Many quantitative data have accumulated about the respiration of brain and nerve. Cerebellar cortex has the highest rate [(9), compare with ascorbic acid content], peripheral nerve the lowest. It is doubtful if any *in vitro* values, on slices or brei, approach the true rates *in vivo*. Values of Q_{O_2} (cmm. O_2 per hr. per gm. of fresh tissue) around 2000 are commonly reported for cerebral cortex of smaller animals; they are found for plugs of cortex with a capillary respirometer (59). With a technique permitting readings within the first few minutes, however, a linear extrapolation to zero time gives values of 5000. To what extent injury has increased this initial rate remains to be determined, but probably it is close to normal. Ringer's solution is at best a poor medium; even nerve respire in serum a third more rapidly than in Ringer's solution, due largely to the presence of proteins (71). The retina is even more affected, the Q_{O_2} in serum being nearly three times that in Ringer, and not depending on the protein factor. Further, in slices and still more in brei, individual ramifying neurones are injured far more than are the more compact parenchymal cells, and brain regions with large cells show greater respiration decreases on mincing than do those with small ones (9).

The respiration of human optic nerve slices is least in the normal, and highest in trunks with marked fiber atrophy (72). The authors attribute this to the activity of proliferating glia, but the same result would also follow if non-neural elements normally had a higher respiration than axones. The respiration of *Limulus* optic nerve, oddly enough, is least at the distal end and greatest in the center (73). A linear gradient in frog nerve of respiration rate, content of fats, salts, etc., is probably related to varying volume relations between axones and other elements (74); a similar explanation may appear for the *Limulus* results.

Metabolism of activity.—The extra metabolism of activity has still to be studied mainly on nerve, due to the difficulties with localization, inhibition, etc., in central structures. Yet some evidence has been obtained of increased heat production in the cat's brain in locally active regions (75, 76) and of increased oxygen usage and ammonia

production by fish brain after excitation *in vivo* (77). The action-heat production of cat's nerve has also been measured and shown to fall into initial and late phases (78). Oxygen consumption studies have led to confusing results. The increased respiration of excited frog nerve or brain does not cover the extra nitrogen and fat metabolized (79) and the conclusion has been drawn that action metabolism is largely anaërobic. For slow molluscan nerve it is claimed (80) that, although ammonia is produced in relation to a period of stimulation, the extra oxygen consumption may not appear until a half hour later. This would indicate that oxidations are concerned only in recovery processes; and an extension of earlier work on the influence of iodoacetate and lactate on nerve conduction in oxygen and nitrogen (81) relegates glycolysis to a similar position.

Still more unexpected is the finding that frog nerve poisoned with yohimbin and stimulated not over five times a second reduces its oxygen consumption but conducts good-sized impulses (62, p. 188). The change is very small and physical effects of the electric currents have not yet been excluded. The author relates the increased negativity produced by veratrin to an increased oxygen usage, and the increased positivity resulting from yohimbin to a diminished one. Such a relation was, in fact, predicted earlier (82) and a double capillary respirometer used to test for an increased respiration in cathodally polarized nerve, and for a decreased one under anodal polarization (83). Large and asymmetric volume changes due to current passage, even with a moist thread replacing the nerve, have so far confused the results. Altered membrane polarization produced by drugs does seem to modify the energy requirement of nerve. [But acetylcholine, which changes the cell potential, at least of muscle, in the negative direction (84), decreases nerve respiration (71).] The further decrease on stimulation of yohimbinized nerve must have a different basis and, if not a physical artefact, may be due to electrotonic depression by the stimulating current. Controls with the electrode portion of the nerve outside the respiration chamber or cut from the main conducting nerve length should decide these points. The finding of a greater respiration of the retina of the frog (85), rabbit and monkey (86) in the dark than during illumination is interpreted in terms of an oxidative reaction accompanying dark adaptation; but even in terms of resting and active metabolism the result can be reconciled with the demonstrated presence of receptors which discharge only in the dark (87).

Respiration under drugs.—The effects of many agents on isolated brain or nerve respiration have been studied. Frog-nerve respiration is 98 to 99 per cent inhibited by cyanide when this is introduced into the manometer cup by a special stop-cock well (88); but a new determination of the inhibition of cortex gives 90 per cent as the maximum (89). Arsenite blocks nerve dehydrogenases (38, 90) and conduction fails when respiration is 50 to 80 per cent inhibited (90). Sulphydryl compounds partially protect conduction with little influence on respiration. Cysteine at first accelerates and later depresses brain oxidations with a lowering of the R.Q. to 0.6 (91). Nicotine largely inhibits lactate oxidation by normal or diabetic brain, while affecting little or not at all the oxidation of pyruvate or glucose (92); hydroxymalonate has a similar action (93). These results indicate that lactic acid is not a necessary step in glucose oxidation. The action is presumably on the lactic dehydrogenase. Nicotine interferes also with succinate oxidation, hydroxymalonate with anaërobic glycolysis and the activating effect of pyruvate thereupon. Novarsenobenzol increases respiration of muscle, etc., possibly by forming arsenate and so increasing lactate (94). Since brain respiration, in contrast, is decreased, some additional non-carbohydrate respiratory system in this tissue may be depressed. 2,6-Dichlorophenolindophenol and 4,6-dinitrocresol increase brain respiration in small concentrations, depress it in stronger ones (95). Dyes influence respiration and aërobic glycolysis independently (see later). Morphine, codeine, cocaine, cobra venom and deuterium water (96) are reported as slowing the reduction of methylene blue by brain brei (compare 49). Light leads to the photodynamic oxidation of proteins in eosine-stained nerve (97).

Ammonia.—Ammonia is formed in isolated brain by the deamination of butyl-, amyl-, isoamyl- and heptylamines (98). Acetoacetic acid is also formed from the isoamylamine in its oxidative breakdown, yet the oxygen consumption of brain cortex (in contrast to that of other tissues) is decreased by the higher amines. Ammonia production is increased during stimulation *in vitro* [0.4 mg. per gm. per hour for *Anodonta* nerve (99)] or following stimulation *in vivo* [fish brain, following dyspnea due to continued partial asphyxia (77)]. A discussion of nerve and muscle ammonia is included in a general review of this substance (100). Recent work has shown a characteristic glutaminase to be present abundantly in brain and retina; it activates reversibly the reaction: glutamine \rightleftharpoons glutamic acid + NH_3 .

l-Glutamic acid doubles the brain Q_{O_2} (with glucose added it is nearly triple) and converts free ammonia to amide nitrogen (101). Only *l*-glutamic acid (of 12 amino acids tried) increases the Q_{O_2} , although the *d*-form also is oxidized by brain extracts (102); one product is α -ketoglutaric acid which is related through diketo adipic and pyruvic acids to glucose. The glutaric or glutamic acids may, by reversible aminization, play a rôle as ammonia carriers in cell reactions.

Substrate and catalyst content.—Before considering the intermediary metabolism of brain, it may be useful to record data on the presence in brain of various catalysts and other significant substances. Glycogen in the cortex of the mammalian brain averages 80 to 100 mg. per cent; in the turtle brain, 300 mg. per cent, as determined by a modified method (103). It is not altered by excess food or starvation, phlorhizin, adrenaline, pancreatectomy or glucose injection even with insulin; but insulin overdosage causes a marked fall (103). This is interesting since the true blood sugar is reduced to zero before insulin convulsions appear (104). It indicates that brain cells deprived of carbohydrate fuel are abnormal and discharge excessively; which is borne out by the direct study of brain potentials under insulin (105) and by the ability of this hormone to decrease the rheobase and chronaxie of nerve (106). The wide, although not yet established, use of insulin shock in the treatment of catatonics and other depressed schizophrenics (107, 108) may derive a rational basis in terms of such findings. Other glycogen values on mammals and pigeons (109) are higher; the cerebrum tends to be low (90 to 130 mg. per cent) and the cord two to three times higher than other brain regions (200 to 300 mg. per cent). Resting lactic acid values, when brain is carefully frozen *in situ* (110), are not above those of plasma.

The content of water, total nitrogen, creatine, cholesterol, saturated and unsaturated phosphatides and cerebrosides in various regions of the central and peripheral nervous structures of a variety of mammalian and other vertebrate brains is reported (111). Conclusions are drawn from these data that the phylogenetically older and less differentiated grey matter (cord) has the lower total nitrogen content; the newer grey matter (cortex) has the higher; and that proteins and other nitrogenous bodies are more vital to function than are the lipoids, with a reverse distribution. Chemical differences between ortho- and para-sympathetic ganglia are also suggested. Creatine is more concentrated in cold-blooded than in mammalian or avian brains, however, which throws doubt on the above generalization. Total

nitrogen and phosphorus increase in fetal brain (human) during the last four months and also show a characteristic distribution (112).

Beef optic nerve has been analyzed for soluble phosphate (25 mg. per cent), phosphorus fractions (phosphagen, 1.3 mg. per cent; adenosinetriphosphate, 1.3 mg. per cent), creatine (about 55 mg. per cent), and creatinine (0.8 mg. per cent) (113). Similar data on lobster and crab nerves and ganglia (114) show 40 mg. per cent of total soluble phosphorus, 11 mg. per cent of adenosinetriphosphate, 5 to 13 mg. per cent of arginine phosphate (more in ganglia) and 11 mg. per cent of hexose- and triose-phosphates. Anoxia causes loss of the phosphagen and pyrophosphate, and a gain of the carbohydrate phosphates; in oxygen arginine phosphate is rebuilt with phosphate from the nucleotide, itself phosphorylated in turn from triosephosphate compounds. Creatine phosphate content of dog and cat brain (about 11 mg. per cent) is alike in cerebrum and cerebellum; the substance decomposes with great rapidity unless this tissue is carefully frozen *in situ* (115). It has been obtained, however, from rabbit brain without such precautions and in even larger amounts (74).

Rabbit brain contains 0.3 mg. per cent of flavin (116); human brain contains 0.15 mg. per cent (7). Human brain is relatively rich in copper, 2.5 mg. per cent, and rather low in iron, 0.4 to 1.4 mg. per cent (118). Dehydrogenases for succinate (117, 119), fumarate, malate (120), malonate (117; cf. 38), α -glycerophosphate (119, 120, 121), pyruvate, lactate (117, 119, 120), glucose (9, 119), etc., and the indophenoloxidase (119, 122) are present and in high concentrations relative to muscle. The glucose dehydrogenase is rapidly inactivated when the cell structure is destroyed. Lactic dehydrogenase, and others, require the hydrogen-carrying coenzyme II, which is present in brain (117, 123) as is also coenzyme I [cozymase (123)]. White substance contains 1 unit of cozymase, grey, 3.7, as compared to 8.1 in muscle (7). Brain zymohexase has the same properties as that of muscle (resistance to iodoacetate, fluoride, cyanide, and a temperature of 60° C.; establishment of an equilibrium at one-third triose phosphate to two-thirds hexose diphosphate) but is present in far smaller quantities (124), about one-fifteenth (125). Phosphohexokinase, which gives an equilibrium between glucose and fructose-6-phosphate, is probably present in brain (126). One or more glycerophosphatases, present in rabbit and guinea-pig brain, act on the α -form of the substrate more than on the β -form; pH maxima are observed at 5.2 and 9.1; they require magnesium and are inactivated

by fluoride, iodoacetate, arsenious acid and phlorhizin in descending order (127). Similar data on sheep-brain phosphatase, which is more active on hexosediphosphate than on glycerophosphate, suggest two enzymes active at the different pH's (128).

Carbohydrate metabolism.—Although carbohydrate catabolism in brain does not parallel that in muscle, recent evidence has emphasized similarities rather than differences. Certainly glucose can form lactic acid via phosphorylated intermediates, although it remains doubtful if this is the only or even the main path available.

On the one hand, the presence in brain of glycolytic and phosphate-splitting enzymes similar to or identical with those of muscle bespeaks similar reactions; indeed, many of the individual steps worked out for muscle also have been demonstrated with brain extracts. A macerate of brain or extracts thereof split fructosediphosphate to triosephosphate, change glucose to β -phosphoglyceric acid and this to pyruvic acid, cause α -glycerophosphoric acid to react with phosphoglyceric acid (still more with pyruvic acid) to give lactic acid, and phosphorylate glucose from added glycerophosphate (129). Dialyzed, stale, or fluoride-treated extracts still catalyze the reaction of pyruvic and glycerophosphoric acids to form lactic acid, although unable to form pyruvic acid from phosphoglyceric acid; and a dialysate of muscle fully reactivates the dialyzed brain extract; so that the two tissue dialysates contain equivalent substances (coenzyme). Iodoacetic acid inhibits the reaction between pyruvic and phosphoglyceric acids, as in muscle (129). The data on avitaminous brain, and on pyruvate accumulation with iodoacetic acid and its disappearance with fluoride (32), can similarly be interpreted as due to interference with the respective reactions, pyruvic acid + glycerophosphoric acid \rightarrow lactic acid, and phosphoglyceric acid \rightarrow pyruvic acid. Further, removal of inorganic phosphate (dialysis or calcium precipitation) from cell-free brain extracts seriously disturbs glycolysis and, conversely, heated cozymase (which no longer aids in oxidations or alcoholic fermentations, but still activates muscle glycolysis by serving as a phosphate carrier, like adenosinetriphosphate) or the adenylic phosphates are able to phosphorylate carbohydrate intermediates and activate glycolysis in these extracts (130).

On the other hand, although the rate of lactic acid formation in brain or its extracts exceeds that in muscle, the activity of phosphatases and related enzymes in brain is very small compared to muscle (7, 124). These enzymes may be rather concerned with still

unexplored reactions in the case of brain. Indeed, such an elaborate series of separate steps (many of which are alternate to others) has now been demonstrated in muscle (131, 132) that, even with all the same partial reactions, two tissues might carry on glycolysis along quite different lines. In any event, many metabolic properties of brain are distinctly different from those of muscle (see previous reviews and earlier sections of this one) and, particularly, glucose rather than glycogen is the favored substrate (32, 130). Lactic acid formation from glucose may pass over the triosephosphate route, being catalyzed by adenylypyrophosphate, or over the methylglyoxal route, requiring reduced glutathione; glycogen follows the former only (133). Lactic acid formation by brain in the absence of inorganic phosphate (borate buffer plus calcium; contrast 130) is not affected by adenosinetriphosphate but much accelerated by reduced glutathione. Finally, brain forms lactic acid from methylglyoxal four times as rapidly as from glucose, so that the glyoxal could be an intermediate, whereas muscle glycolyzes glycogen five times more quickly than methyl glyoxal (133). Also the loss of α -glycerophosphate added to brain is not increased by added pyruvate nor, in beriberi brains, by vitamin B₁, as might be expected for the Embden-Meyerhof sequence (121).

Glucose is the normal fuel for brain oxidations, but lactic acid is easily burned, and possibly galactose also (134). Though lower values have been reported (135), the R.Q. of grey matter is 1.0 under a great variety of conditions (136). Lactate is not an intermediate in the oxidation of glucose (see earlier; also its aërobic removal is slower than its anaërobic production), but it reaches the brain via the blood and, when present in excess *in vitro*, may completely supplant other fuels; the constant rate of lactate disappearance accounts, if the lactate is fully oxidized, for the maintained oxygen consumption (137). Recent work has emphasized the interrelations of carbohydrate metabolism not only with phosphates but with succinic acid, acetoacetic acid and ammonia as well. The suggested rôle of succinic and fumaric, or fumaric and oxalacetic, acids as a hydrogen-carrying link in the catalytic chain between activated substrate and oxidized cytochrome (138), is supported for brain to the extent that malonate inhibits glucose (but not pyruvate) oxidation and that fumarate partly reverses this inhibition (139). With brain extracts, however, lactate oxidation is not furthered by the presence of succinate nor does excess lactate prevent the oxidation of

added succinate (119). Fatty acids are not oxidized to acetoacetic acid by brain, in contrast to several other tissues, but β -hydroxybutyric acid is oxidized (140). Acetoacetic acid, as hydrogen acceptor, in turn may play an important rôle in a reaction sequence converting pyruvic acid into succinic acid (141). Two pyruvic acid molecules dismute anaerobically (but see 35) forming acetic acid. This plus pyruvic and acetoacetic acid yields α -ketoglutaric and β -hydroxybutyric acids. The ketoglutaric acid is reduced by another acetoacetic acid molecule to form succinic acid. With the hydroxybutyric acid directly oxidized back to acetoacetic acid, a new redox cycle is interposed in the sequence of carbohydrate oxidations. Vitamin B₁ is the coenzyme for these dismutation reactions (141). The relation of α -ketoglutaric acid, in turn, to glutamic acid, the ammonia metabolism, and the oxygen consumption of brain have already been considered.

The Pasteur reaction is no longer accepted as a quantitative relation between the oxygen consumed and the lactic acid produced, at various levels of oxygen consumption, for evidence has appeared that the oxygen tension, rather than consumption, is related to glycolytic rate (142). The balance between oxidized and reduced forms of a glycolytic catalyst might thus be the determining factor and, for brain, glutathione may play this rôle (132). It is also possible that permeability changes, affecting the availability of glucose to cell enzymes, are involved (143, 144). At least it is established by the action of dyes that aerobic glycolysis and respiration can be independently altered (95, 136), and phenosafranine specifically inhibits the Pasteur reaction (136), as does potassium (with additional complications) (145). The Pasteur effect involves a decreased catabolism of sugar in the presence of oxygen as compared with its absence, and quantitative data on rabbit cortex (137), although few, support the view that the oxidation of carbohydrate alone is concerned in the Pasteur reaction and that brain oxidizes carbohydrate only. At high temperatures (42 to 45° C.), the control of carbohydrate metabolism breaks down and aerobic glycolysis of the rabbit cortex greatly increases despite an increased respiration (137). Consciousness is lost at 42° C. The use of hyperthermia in neurosyphilis, etc., like hypoglycemic shock in schizophrenia, may thus depend on a modification of the carbohydrate metabolism of the brain.

Salts.—The content and influence of salts on neural structures have been intensively studied. A lack of sodium depresses nerve

respiration as does excess potassium. A relatively small calcium increase or decrease has an inverse effect on oxygen consumption (146; but see 147), and magnesium can substitute for calcium. Decalcifying salts all increase metabolism and irritability, even to spontaneous repetitive activity (148). Anions and cations antagonize, and in general produce effects on respiration parallel to those on colloid dispersion. No salt effect has been found on creatinephosphate in nerve (74), although a distinct one is present in muscle. Brain respiration is markedly increased for an hour or more by potassium salts (145) [and those of other alkali metals (149)] as is aerobic glycolysis, while anaerobic glycolysis is depressed. Respiration varies inversely with the calcium content, as in nerve, and diminished calcium or increased potassium causes prolonged after-discharge (150) and spontaneous activity (62, p. 292; 151, 152) of motor centers; excess calcium has the reverse effect (150, 153). Magnesium also depresses brain respiration (149; but see 146). Other relations between salt composition and the oxygen consumption and glycolysis of brain have been studied and many of the effects found to be unique to this organ (149). Acid increases the discharge of ganglion cells of *Limulus* heart (154) and of nerve (155), very possibly by inactivating as phosphates the contained calcium. The presence of 6 mg. per cent of oxalic acid in cerebrospinal fluid (156) is of interest in this connection. Reported values of other ions in cerebrospinal fluid in mg. per 100 cc. are: magnesium, 2 to 3 (157, 158); sodium, 340 (159) or 440 (74); calcium, 6.5 (158); chloride, 415 (158) or 470 (74); potassium, 12 (74). The potassium content is high in the central nervous system, 300 to 400 mg. per cent in various parts (74, 160); it is lower, 220 (160) or 135 mg. per cent (74) in the sciatic. The chloride content of brain in various diseases (161) and the mineral distribution within cells (162) have also been studied. Normal values are 125 to 150 mg. per cent for various brain parts, 200 mg. per cent for nerve (74).

An especial significance of salt ions in relation to membrane potentials and stimulation has long been recognized and they are probably also vital in setting up the metabolic changes attendant on nerve activity (62, p. 194). An additional interest has recently accrued to the action of salts since they are so intimately bound to the problem of neurohumoral mechanisms. Thus, for the cat's superior cervical ganglion, potassium is lost when the cells are stimulated reflexly (163); conversely, injection of potassium liberates acetyl-

choline (164) and increases the response to preganglionic impulses (excess, of course, blocks) or initiates cell discharges. At the neuromyal junction, transmission is aided by increased calcium (165, 166, 167) (or parathormone, 167; but see 168), which can overcome block by fatigue and other curare-like conditions. Potassium has a reverse effect (165; but see 168. Also see 62, p. 347, on receptor organs). Potassium also raises the temperature for cold block of nerve (169). It is reported, finally, that migration of potassium to the cathode, and of calcium to both cathode and anode (due to a negative calcium protein ion) of a polarized nerve occurs only when the nerve is in the normal irritable state and not when it is killed or even partially anesthetized (170). Previous claims of ion migration under physiological conditions have not been substantiated; nor would a 120 m.a. polarizing current leave a nerve physiological.

Neurohumors.—In the past two years over two hundred papers have been directed to the study of neurohumoral substances and mechanisms and, although properly an aspect of nerve metabolism, this field can be but touched on here. Fortunately, many aspects have been thoroughly discussed in the recent symposium already referred to (62, pp. 111, 132, 143, and 358; see also 171, 172).

The liberation of acetylcholine at many autonomic effectors, and of sympathin (173) or adrenaline (174) at others, is now well established, although even for the classic case of the heart, work on fish embryos suggests that acetylcholine is liberated at intramyocardial synapses rather than at the postganglionic endings (175). The liberation of acetylcholine on indirect stimulation of skeletal muscle, the twitch-like mechanical and electrical responses to rapid acetylcholine injection (176; but see 177), the decurarizing effect of prostigmin in myasthenia gravis and other paralyses (178), and the facilitation of fatigued (179) or curarized (180) muscle for minutes after a brief tetanus to its nerve, all favor the participation of a similar chemical mediator in neuromyal conduction. In a variety of autonomic innervations, the nerve or its mimetic drug that evokes positive responses gives negative potentials, and the reverse is true for inhibitory nerves and drugs (62, p. 111); so that the humoral agent in each case may act by lowering the membrane potential of the effector cell to evoke excitation, by raising it to inhibit. The depolarizing influence of acetylcholine on skeletal muscle, stopped by curare (84), is quantitatively sufficient to cause contraction, in good accord with this view. In fact, evidence on nerve (181), ganglion (182), and

brain (62, p. 320) also points to a general parallelism between lowered membrane potential (negative potential waves) and lowered excitation threshold, and between positive waves and depressed irritability.

Besides the mechanism of the action of neurohumors, those of their formation and liberation are important. It is almost necessary, from metabolic considerations, that ion changes set up by a nerve action precede and control the release of mediator (62, p. 194), and evidence on the relation of potassium to acetylcholine does point this way. Whether the special humor is freed only at modified terminals of a nerve fiber or, like the salt changes, appears throughout its length, is still debated. Dog vagus, stimulated *in vivo* or *in vitro*, liberates a heat-stable substance which sensitizes leech muscle and rabbit gut to acetylcholine (183). Spectroscopic evidence suggests this may be a cyclic compound (184). Glucose inhibits its liberation (185). The claim that vagus and sciatic nerves of a variety of animals produce a cardio-inhibitor substance at the cut end of the central portion as centrifugal impulses reach it, and a cardio-accelerating agent at the proximal cut end of the peripheral portion with centripetal impulses (186), makes one welcome a control (187) indicating that humoral agents are liberated from nerve trunks only in the interelectrode stretch and on passage of excessively strong stimuli. A seemingly crucial experiment, however, is the accumulation in eserinated Ringer of acetylcholine (leech test) from the cut end of the central stump of the dog's phrenic nerve, only so long as the regular respiratory impulses are being conducted (188). No acetylcholine accumulates during a period when impulses do not travel, due to cold block of the nerve or to apnea of the centers (over-ventilation). These experiments should be repeated. The reported presence of a special convulsive agent in brain extracts, also a hypnotic one from sleeping brains (172), has not been confirmed (189); but a vasodepressor protein from brain (190) and a vasopressor substance in the cerebrospinal fluid of hypertensive patients (191, 192) seem to be real.

The formation of neurohumors is almost surely in the structure that eventually liberates them. Choline and its ester are normally absent from the cerebrospinal fluid (193, 194; but see 195, also 196 which describe its accumulation under eserine and asphyxia or adrenalin) but are richly present in brain and especially nerve. In $\mu\text{g. per gm.}$, vagus contains 5, sciatic 2 (194, 195, 197), spinal cord 0.5 to 1 (195, 197) or 4 (194), cerebral white matter 0.2 to 6 and grey matter 0.7 to 2 (197), the optic thalamus being richest. Liver and muscle

contain about 0.1 $\mu\text{g.}$ per gm. (195). Choline and esterase distribution do not parallel that of acetylcholine (195), although the esterase is also especially rich in the basal grey matter (198). Morphine markedly inhibits esterase activity (199). The perfused cervical ganglion gives off 0.8 $\mu\text{g.}$ (estimated) of acetylcholine during two hours preganglionic stimulation without the amount extractable from the tissue being diminished (164); this is direct evidence of its synthesis. The ester is formed (biological tests) by brain slices in eserinizied phosphate buffer, more in the presence of glucose, almost none in the presence of cyanide or absence of oxygen (200). In three hours ten times the amount initially present can form; it is clearly produced by the active metabolism of brain cells. The synthesis of adrenaline, *in vitro*, by kidney cells has also been reported (201). It can hardly escape notice that the lipids of myelin, if concerned in active metabolism, would be likely choline sources, and a choline ester of sphingosinephosphoric acid has been reported (202).

If the liberation of neurohumors from nerve is uncertain, that from brain is more so, and the rôle of chemical mediators in central conduction is not established. Cerebral embolus causes the liberation of a histamine-like substance from the brain (203, 204). Reports of acetylcholine (205) or locus-specific neurohumors (206) in the effluent blood from medulla or cerebrum are not lacking. This ester is also found in fluid about an isolated spinal cord when it is stimulated and is credited with producing the chronaxie of subordination (207). Acetylcholine injected into the hypothalamus of cats causes sleep and a fall of blood pressure; pilocarpine excites (208, 209). Applied to the floor of the fourth ventricle, it causes hypertension and hyperpnea (210). Conversely, acetylcholine prevents the convulsions of camphormonobromide (211). Ergotoxin, intravenously injected, produces rage in cats, possibly by cerebral depression (212), and ergotamine is effective in migraine headaches (213).

Ultrastructure.—An interesting start has been made on the study of nerve ultrastructure by the use of x-rays, polarized light (62, p. 7) and thermal shortening (214). Diffraction patterns depend rather on radially oriented lipid micelles than on longitudinally oriented ones of protein. Lipoids and proteins, as oriented, lead to opposed birefringence changes, and axone birefringence varies with fiber diameter so that these effects balance at 2 μ , which is also the radius for transition from medullated to non-medullated fibers. Conduction velocity in large invertebrate nerves, and their birefringence, fit a

diameter of 2 μ , rather than the actual one of 50, so membrane ultrastructure may be vital to conduction properties. Four nerve proteins have been extracted at high pH's (215). A protein-lipoid colloid of brain with isoelectric point 4.4 to 4.6 binds considerable fixed base at body pH (216) and contains active oxidizing enzymes (119).

Finally, the great body of work upon non-chemical attributes of brain and nerve, especially the electrical phenomena, and even studies on the action of many chemicals upon these other properties have been largely ignored. The symposium volume already referred to (62) fortunately provides a convenient key to this literature.

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¹ Since over 400 articles touching on the chemistry of brain and nerve have appeared in the past two years, this bibliography makes no pretense to completeness. With rare exceptions, work prior to 1935 is not referred to; it is available for comparison in preceding reviews in this series and in volume 12 of *Physiological Reviews*. Papers which have appeared in the review period, if adequately referred to in later articles here listed, have also often been omitted from this bibliography although considered in the text. Still other articles have not been considered, due to space limitations or oversight. I am indebted to Mrs. Mabel Blake Cohen for her loyal assistance.

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THE BIOCHEMISTRY OF FISH*

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The technical literature in the field of fish culture is very dispersed. In America much of it appears in special bulletins issued by federal and state bureaus. In Europe it is found in technical journals of limited circulation. In America the seven hundred or more fish hatcheries are kept well abreast of scientific advances by mimeographed material issued at frequent intervals by conservation departments and the United States Bureau of Fisheries, but the annual volume of the Transactions of the American Fisheries Society which links the laboratory and the fish hatchery is available in only a limited number of libraries.

In considering the biochemistry of fish two facts must be kept in mind. In the first place, there is a great species difference. The carp or goldfish is as different from the trout in its nutrition as the cat is from the cow; this restricts generalizations. In the second place, the most widely studied species, the trout, conduct their life processes at very low temperatures. Most studies with trout that will be included in this review were made at body temperatures of approximately 8° C. A unique condition has thus evolved in which certain species, the trout, die if the body temperature exceeds 15 to 20° C. for any long period of time. This temperature condition has led to the many classical discussions concerning the identity of such enzymes as pepsin in trout and in the higher warm-blooded species.

CHEMICAL EMBRYOLOGY

Needham (1) summarized the literature in regards to the chemical changes in fish eggs during hatching. Novak (2) has confirmed some of the earlier work in his recent study of carp eggs. During hatching the water content tends to remain quite constant. There is also little loss of nitrogen until the time of hatching when the shell is dropped. Dry matter decreases about 6 per cent. Individual eggs vary widely in lipids; one may have three times as much as another. These data

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indicate that part of the metabolic products do not leave the egg during hatching. Lovern (3) has followed the changes in the fat of ripening salmon eggs.

The production of monsters as a result of certain chemical constituents in the water surrounding hatching eggs is a well-established phenomenon. Surber (4) has reviewed the literature in regard to the effects of carbon dioxide and has shown experimentally that amounts of 55 to 78 p.p.m. of carbon dioxide tend to cause heavy losses during the post-eyed stage.

The "sac fry" stage in the life of a fish is the period immediately after hatching from the egg, during which the minute fish depends for its nutrition upon the materials stored in the "sac." During this period the fish body gradually consumes the compounds of the "sac" and at the end of the period the fish attains the "fry" stage and starts eating. Novak (5) has followed the changes in composition of the carp embryo during this period by fixing specimens in formaldehyde and then dissecting the body from the sac. In the course of five days much of the fat, nitrogen, and ash moves from the sac into the body. However, there is some evidence that about the third day some of the nitrogen is excreted from the body back into the sac. Calcium and phosphorus tend to increase slightly in trout eggs from the time these leave the body of the fish until the fry begin to eat (6). Sekine (7) found an increase in the lipid-phosphorus compounds and the ash constituents of salmon eggs during hatching. The young salmon fry were able to assimilate some of the inorganic matter of the surrounding medium.

GROWTH

The growth of trout has been extensively studied during the past ten years. Trout tend to grow logarithmically and at a slower rate than warm-blooded animals according to the findings of Titcomb *et al.* (8, 9). Four species of trout, lake trout (*Cristivomer namaycush*), brown trout (*Salmo fario*), rainbow trout (*Salmo irideus*), and brook trout (*Salvelinus fontinalis*) were fed a similar diet and kept under identical water conditions from the time of hatching until four years of age. Under natural conditions fish tend to grow rapidly during the warm months of summer and slowly during the cold winter months, but under conditions of constant water temperature these four species of trout grew at almost identical rates (6). The growth

rates tended to be constant for many months each year, but each year the rate was less than in the preceding. The growth rate tends to decrease during the period of spawning. The gradual decline in the growth rate of trout during a period of years indicates that this growth is determinate although it may continue at a constantly decreasing rate for many years.

Trout react similarly to other species by ceasing to grow when the diet is deficient in quality or quantity. After a period of twenty weeks of retarded growth as a result of inadequate calories, trout were able to compensate by means of very rapid growth even when subjected to decreased water temperatures (10). After periods of retardation trout tend to exceed the normal growth rate (11). In the case of carp, Matsui & Oshima (12) have attempted to employ alternate periods of underfeeding in order to secure efficient conversion of foodstuffs. Trout can live several months with no food (8). For this reason nutrition experiments must be run for relatively long periods before specific deficiencies are developed (8).

The rate of growth of trout in hatcheries is much more rapid than that in streams due to many factors such as more favorable food supply. The belief is quite common that the trout produced under such conditions are weaklings and tend to undergo a heavy mortality when set free in streams (13). This is only a special case of the many claims in both the technical and popular literature that rapid growth produces animals that cannot endure unfavorable environments as well as those that mature slowly. Experimental evidence that favors this theory has come from experiments in which trout were subjected to common vitamin-like deficiencies but in which some groups were prevented from growing by a low protein level in the diet (11). Thus, trout that did not grow, or grew very slowly, lived nearly twice as long as those that grew normally. This led to the thesis that the trout body contains some constituent that is consumed more rapidly during growth and if this constituent is not provided in the diet the death rate tends to rise more rapidly than in the case of retarded animals (8). Many types of unfavorable environments need to be tested, however, before this relation of growth rate to resistance is established.

Old carp are able to fast much longer than younger ones and during such periods the parts of the body are sacrificed at unequal rates since the head tends to be preserved (14). Nowak (15) has given special attention to the changes in the chemical composition of carp fry during the earlier period of their growth.

UTILIZATION OF DISSOLVED NUTRIENTS

The utilization of dissolved nutrients by fish has been debated since the time of Pütter. Krizenecky (16) reviewed the evidence in 1925. Upon the basis of experiments with tadpoles he concluded that nutrients such as glucose could be absorbed from the water. There were three possible channels for this absorption: the skin, the alimentary canal, and the gills. In fish the absorption seemed to take place through the gills while in tadpoles there may have been some through the skin also. Chomkovic (17), from studies with fresh fish skins, concluded that these were semipermeable and that this afforded a means of taking nutrients from the water. Podhradsky (18) found that potassium and calcium ions affected the growth of tadpoles kept in nutrient solutions. Calcium chloride in 0.2 per cent solution stimulated growth, while higher concentrations retarded it; both potassium and calcium ions caused retardation. Kostomorov (19) found that carp could utilize dissolved nutrients but could not maintain their body weights. Krogh (20) found fish and tadpoles could utilize dissolved glucose to satisfy as much as one-fourth of their energy requirements.

In solutions of glucose (1 to 5 per cent) carp may double their blood sugar in the course of fifteen minutes (21). This indicates quite rapid absorption. The exchange of water between the environment and the body of fish was found to be very rapid in the case of goldfish (22). This was determined by immersion of individuals in water containing known concentrations of heavy water. The rate at which equilibrium was established was much more rapid in the case of live fish than in that of dead ones.

Recent experiments by Schuster-Woldan (23) indicate that fish may actually drink nutrient solutions. Tench (similar to the familiar chub) and carp were photographed after immersion in barium sulfate suspensions with and without glucose. The x-ray showed that considerable amounts of the suspension had passed into the intestinal tract when there was glucose in solution but none in its absence. Smith (24) found that marine fish swallowed large amounts of water.

INORGANIC NUTRIENTS

Practical experience has long indicated that inorganic constituents are absorbed from the water. Carp are known to have more calcium in their bones, as well as more ash, if they originate from

hard waters, even if those from the softer water have the same diet. Such evidence is never conclusive, however, because a certain amount of living material is contained in the diets of such carp. It has also long been known that trout do not develop enlarged thyroids in water containing iodine.

The source from which fish derive their body calcium is a problem of special interest because the diets employed in many of the trout hatcheries during the past fifty years have consisted largely of meat, especially beef liver. Some hatcheries have soft water supplies with little calcium and others have very hard water. Upon these meat diets which are rich in phosphorus and poor in calcium one might expect deficiencies in the bones. Analyses of entire trout bodies reared upon liver alone or liver supplemented with a rich source of calcium, such as ground fish bones or dried skimmed milk, yielded values that were approximately the same, indicating that the trout were probably obtaining an ample supply of calcium from the water (25).

Analyses of trout before and after a feeding period of ninety days, indicated that each trout acquired about 100 mg. of calcium from the water and only about 30 mg. from the food (26). In a later more comprehensive study the changes in both the calcium and phosphorus were followed from the time eggs were taken until the trout were well advanced in the fingerling stage (27). Calcium was removed from this water which contained 44 to 46 mg. per liter. As soon as the young fry start to feed the increase in body calcium becomes marked. However, it requires many months before the calcium equals the phosphorus, thus establishing the ratio that persists thereafter in the body of the adult fish.

In young salmon kept without food immediately after absorption of the yolk sac, Sekine (7) found that inorganic matter increased from the water, especially when the fish were kept in the dark.

All recent evidence indicates that fish can remove essential materials which are in solution and which they need for their metabolism. Thus far no one has attempted studies with solutions that represent even approximately complete diets.

ULTRAVIOLET LIGHT

Little is known concerning the effect of light upon the metabolic processes in fish. Both trout and carp are very sensitive to ultraviolet irradiation (28, 29, 30). Thus, trout in water 2 cm. deep were de-

stroyed by five daily exposures of three minutes each. Kostomarov (30) found that carp were readily checked in growth and their bodies lost both organic matter and ash. In small amounts, however, the ash constituents of the carp body seemed favorably affected although no data were presented to support this conclusion. Some of the external parasites of trout can be destroyed by irradiation but this treatment has not been studied sufficiently for use in practice.

The effect of ultraviolet light upon the eggs and sac fry of three species of trout, as well as pike (*Esox lucius*), was studied by Haempel & Lechler (29). They found the eggs were especially sensitive and easily destroyed. Strong irradiation of eggs seemed to modify both the egg sac and the embryo after hatching. Freshly fertilized eggs seemed more resistant to unfavorable effects than "eyed" eggs. Trout species seemed to differ in sensitivity, and brook-trout eggs were especially affected.

METABOLISM TECHNIQUE

In conducting nutrition experiments with fish, technical improvements have insured more reliable results during the past few years (31, 32). In the first place modern hatchery construction provides pure water that is quite constant in temperature and chemical composition. Individual troughs are provided in experimental hatcheries, such as that at Cortland, New York, with separate water supplies from the original source. Much of the early nutrition work with fish was confused due to the washing of food residues and excreta from one trough to another. Chemical balance work has also been improved by the use of air under constant pressure from installations such as those employed in garages. Air is diffused through the water by the use of tips of porous carborundum. The temperature of the water used in balance trials is maintained by the immersion of glass aquaria in the flowing spring water of the hatchery. Unfortunately few universities have facilities for the study of such species as trout that are very sensitive to temperature and the quality of water.

SYNTHETIC DIETS AND VITAMIN NEEDS

Synthetic diets of partly purified constituents have been used to a limited extent in attempts to define the nutritional requirements of fish. Trout can make moderate growth for periods of three months

upon diets of casein, starch, fat, inorganic salts, and the usual vitamin supplements used for rats (33, 34). However, at the end of such periods they die unless special supplements of fresh meat or extracts are added to the diet. Jewell and coworkers (35) have used such diets for goldfish and catfish. The unique character of the vitamin requirements of fish as well as many other lower species has tended to block progress in defining nutritional requirements.

The unsatisfactory nature of the evidence that fish need any of the recognized vitamins was reviewed in 1927 (33). Until that time the work of Davis & James (36) had provided the best evidence that carp and possibly trout needed vitamins A, B, and C. At that time McCay & Dilley (37) found that trout could live and grow for about three months upon synthetic diets of casein, starch, lard, mineral mixture, cod-liver oil, and yeast. At the end of this time death ensued unless the diets were supplemented with fresh meat. Similarly trout could be reared upon mixtures of dry skimmed milk and fresh liver, while they died if fed dry skimmed milk supplemented with such vitamin-rich products as cod-liver oil, "Vitavos," or inorganic elements such as iron and copper (11). On the basis of such experiments it was recognized that trout required some thermolabile fraction present in fresh meat and different from any of the recognized vitamins. This was termed "factor H." In many respects this thermolabile factor resembled that which Wulzen (38) had recognized in her studies with planaria. Since the beginning of the hatchery rearing of trout the practical fish culturist has recognized this constituent as the dominating factor in his choice of diets. Therefore, while many other cheaper materials, such as cereal grains and legumes, had been tried in the trout hatcheries, they were always fed in mixtures containing fresh meat. Today the hatcheries of the United States feed about 4,000 tons of fresh meat compared to 400 tons of plant and dairy products, according to the survey of Fiedler & Samson (39).

Further evidence concerning the nature of factor H was presented by Titcomb *et al.* (40). It was discovered that meat preserved in alcohol (41) retained its potency. Meat dried *in vacuo* under carbon dioxide and preserved under this gas retains its potency (25). Meats also tend to retain their potency when preserved with formaldehyde or hypochlorites (31).

Heard (34) found that extracts of liver made with alcohol, ether, and acetone stimulated the growth of trout for short periods. Greenberg & Schmidt (42) have attempted the isolation of the factor in

liver that stimulates the growth of planaria. They have concluded that it is heat-labile, ether-soluble material, destroyed during saponification and not identical with the phosphatides or non-saponifiable lipids of liver. Hewitt (43) has concluded that factor H consists of one or more substances similar to lecithin in composition.

The use of fresh and autoclaved spleen for supplementing diets fed to rainbow trout has been studied by Haempel & Peter (44). They found that raw spleen and cattle stomachs are adequate for the growth of trout, but if this mixture is autoclaved the trout cannot grow, and die. The addition of cod-liver oil, yeast, and carrot juice did not provide the factors destroyed in the autoclaving. In earlier work, Haempel (45), employed various mixtures of casein, albumin, yeast, and other supplements but these diets failed unless they contained raw spleen. Yeast seemed to have some function, however, inasmuch as it prevented injury to the gills by a basal diet of autoclaved meats. Probst (46) also studied spleen in the diet of rainbow trout. He found they failed quickly upon dried spleen but if this were supplemented with fresh spleen, growth and survival were normal.

Some of the early German workers seem to have held the view that natural live forms were essential in the diet of carp to furnish enzymes for the digestion of plant materials. The evidence behind this theory, which seems to have originated in the laboratory of Zuntz, is unknown.

Jewell and coworkers (35) ran preliminary trials to determine the vitamin needs of channel catfish (*Ictalurus punctatus* Raf.) and goldfish (*Carassius auratus* Linn). Goldfish, fed diets containing cod-liver oil, showed improved skeletal development. The authors concluded that both species studied required vitamin D. They could get no conclusive evidence concerning B factors, although the goldfish which received the diet lacking vitamin G seemed more susceptible to fungus growths. Raw meat stimulated the growth of catfish much more than any other vitamin supplements. Goldfish did not react appreciably to the raw meat, thus showing a difference between these species. Yeast stimulates the growth of trout fed synthetic diets (41).

Mottram (47) made x-ray photographs of trout reared in hatcheries upon horse meat and those living in streams under natural conditions. He found that skeletons of the wild trout more dense and inferred that the hatchery fed trout may have been suffering from a calcium deficiency. This hatchery, however, had chalk-stream water and it is not likely that the differences found were due to a calcium

deficiency. Some such factor as growth rate is the more likely reason for this difference.

Ueno and coworkers (48) have suggested that carp be used for the assay of vitamin A. They find, however, that the fall of water temperature in the winter complicates the assay.

Most of the recognized vitamins are present in various organs of fish so we may infer that these vitamins function as they do in higher animals. At the same time the great variability in the concentration of the fat-soluble vitamins in the liver oils of different species indicates some of the differences that can be expected in nutritional requirements (49). Recent studies of the vitamin-C distribution in the bodies of fish indicate that it is especially high in the roe, brain, and lens (50). Fish seem to have a distribution of vitamin C similar to that found in other animals (51).

The conditioning of water by a given species is a phenomenon of interest to students of growth in fish and other lower forms that live in water. Evans (52) has fed goldfish synthetic diets with and without vitamin supplements of yeast, lemon juice, and halibut-liver oil. Those in the conditioned water grew better. Survival was greater in the groups which received the supplements. Goldfish could grow for about sixty days without vitamin supplements. Rat tests showed that the stimulating effect of the conditioned water was not due to any of the recognized vitamins.

PIGMENTATION

The problems of the pigmentation of fish remain unsolved although recent advances in the vitamin field are suggestive. The fish culturist has been concerned with these problems since the domestication of trout began. No one has discovered methods of producing trout with the same brilliant colors that are found in nature. The great variation found among wild trout in the same school has led to the belief that an inherited as well as a food factor is involved. Lönnberg (53) recognized four classes of pigments: (a) lipochromes contained in the xanthophores and erythrophores; (b) guanine in special cells; (c) melanin granules held in the melanophores; and (d) pigments distributed generally throughout the body. Lönnberg has extracted the fat-soluble pigments from several hundred species of fish, birds, and lower vertebrates and examined them spectroscopically. The studies of Drummond and coworkers concerning the syn-

thesis of carotenoid pigment by young rainbow trout were reviewed in an earlier volume (54).

In 1930, Davis (55), at the Pittsford hatchery, found that trout fed dried salmon eggs "showed the brilliant coloration of wild trout of the same age." Later experiments have failed to yield the same results as those of Davis (26). Some pigmentation has been produced, however. The greatest difficulty has been to secure dried salmon eggs of good quality. Attempts to pigment trout by feeding concentrated extracts of salmon eggs have yielded variable results. Mann (56) finds that trout can be pigmented by feeding them goldfish, carrots, daphnia, and certain other natural foodstuffs. He believes that the fat-soluble pigments originate in the foodstuff. Food is also the important factor in the pigmentation of pike, according to Scholz (57). Salmon oil produces some pigmentation. Sudan III and IV color the mesentery fat but not the external cells of trout (31).

Euler and coworkers (58) found that predatory fish could not transform carotene into vitamin A but that plankton feeders could. Salmon muscle yields astacin (59, 60) as well as xanthophyll and carotene (58). Astacin has also been isolated from the eggs of cod (61). Sumner & Fox (62) fed diets with and without xanthophyll and carotene to *Fundulus parvipinnis* G. This species seemed able to convert carotene to the xanthophyll found in their bodies. Sumner & Fox also found (63) that another species, *Girella nigricans*, lost 85 per cent of its color in five months and this loss was not checked by feeding. Vitamin A, carotene, xanthophyll, and lactoflavin have all been found in the eyes of fish (64, 65).

FAT METABOLISM

The digestion and absorption of fats by fish have been subjects of considerable experimental work since the later years of the past century. Studies with carp in warm water by the usual techniques early indicated that there was a close correlation between the food fats and those deposited in the body. From the time of Knauthe it was commonly believed that much of the body fat of carp originated in the feed. Mieller (66) has recently compared the iodine numbers of the body fats of fish with the mean values for the most common natural foodstuffs. Thus he finds that brook trout have values of 124 and 110, respectively, for body fat and foodstuff, while pike show values of 54 and 79. The greatest error in such studies is the assigning of equal weights to each class of natural foodstuff ingested.

In a series of studies similar to the early ones of Rosenfeld, Lovern (67) has presented additional evidence that the food fat exerts a marked influence on the quality of the body fat both in the case of fresh and salt water fish.

The metabolism of fat constitutes a unique problem in the case of fish species such as brook trout that can only survive in cold water. The emulsification, digestion, and absorption of high melting fats must be difficult under such conditions. This is probably the basis of the common prejudice of the fish culturists against fats, whether they originate from mutton, pork, or beef. The iodine numbers of the body fats of trout from the same group, that have been maintained upon a common diet, may vary from 93 to 119 in one group (25). The value for the body fat in the case of hatchery fed trout reflects that of the diet employed.

The mesentery fat of brook trout tends to reflect the food fat in respect to its iodine number (26). When cottonseed oil was fed as a supplement to the trout diet this mesentery fat had an iodine number of 100 to 109 while the values were 90 to 95 when hydrogenated oil was fed. In balance studies with brook trout the relative utilization of salmon oil, cottonseed oil, and hydrogenated cottonseed oil was determined (31). Groups of trout of a mean weight of 2 gm. were compared with those of a mean weight of 100 gm.; the studies were made at 8° C. The fats were fed at two levels of 7 and 25 per cent of the diet. The first two were used to the extent of 80 to 90 per cent, whereas 15 to 53 per cent of the hydrogenated fat was recovered from the feces. In every parallel trial the oils were better utilized than the hydrogenated product. The size of the trout and the level of fat ingestion made no significant difference. Thus in spite of the very low body temperatures trout are able to partly utilize fats of relatively high melting points. Fish oil is not utilized to any better advantage than vegetable oil. Trout have been fed as much as 57 per cent of their diet in the form of fat without apparent injury (33).

In recent years considerable concern on the part of some of the fish culturists has resulted from their observations that trout tend to develop livers that are excessively fat as the result of overfeeding.

CARBOHYDRATE AND PROTEIN REQUIREMENTS

The part played by carbohydrates in the diets of fish is little understood. There is no doubt that carp utilize starches to good advantage. Early data indicated that better use was made of cooked than of raw

starch by carp. According to Geng (68) some plankton may contain more than 40 per cent of carbohydrates and may comprise the natural foodstuff of even carnivorous fish. Brook trout seem to make better use of cooked starch or dextrin than of raw starch (40). Preliminary studies of the starch balance of brook trout indicate they can make some use of both raw and cooked starch although they tend to excrete some of both in the feces (69). Lactose does not seem to be better utilized than starch (37).

Various attempts have been made to define the protein needs of trout. When casein is the source of protein it must exceed 10 per cent of the calories in the diet or the trout cannot grow (33). Moderate growth results if the casein exceeds 20 to 25 per cent (41). During periods of very rapid growth trout need about 14 per cent of protein in the diet if it is in the form of raw liver (25). Under such conditions, if levels of 6 or 10 per cent of liver protein are fed, growth is submaximal and death follows in ten to sixteen weeks.

Considerable amounts of cellulose are ingested by such fish species as carp. Early workers thought some of this was digested; its fate is uncertain. Schäperclaus claims that even trout need some source of roughage (70). Under natural conditions this is furnished by chitin as well as cellulose. In spite of the very short length of the intestinal tract, or possibly because of it, trout can tolerate very high levels of inert material such as regenerated cellulose (40). At levels above 25 per cent of the diet the growth rate tends to be decreased and at 35 per cent, growth is checked (41). Agar-agar seems to make no improvement in trout diets (37).

SPECIFIC EFFECTS OF COMMERCIAL FOODSTUFFS

Fish display specific reactions to certain of the more common commercial foodstuffs. Cottonseed meal can be fed to trout in an amount equal to about half of the calories ingested. Even at levels of 70 per cent there is no evidence of toxicity (31, 41). Among available foodstuffs of plant origin such as linseed meal, cocoanut meal, and soybean meal, linseed meal is the poorest and cottonseed the best (40). Trout can make use of corn gluten, peanut meal, and wheat flour for growth and maintenance (11). Trout are especially sensitive to some poison in the older forms of linseed meal. The literature in this field was discussed in an earlier article (40). In these experiments trout were fed diets containing 25 per cent of linseed meal. In about four

days they lost equilibrium and stopped eating. After this they revived and consumed more. In four weeks all were dead. Bullheads (*Ameiurus nebulosis*) fed linseed meal fail to show any toxic reactions (25). Boiling linseed meal does not remove the toxic effect (41). It has been suggested that fresh meat contains an enzyme which sets free hydrocyanic acid in the stomach of the trout, but preliminary experiments fail to show this. Carp do not seem to be poisoned by linseed meal (71).

Milk by-products have proved especially valuable in the trout hatchery. Trout can grow satisfactorily upon dried skimmed milk alone for several months (33). Combinations of this product with raw liver provide complete diets for trout. Dried buttermilk is equally satisfactory. Dried skimmed milk is not rendered complete, however, by supplements of vitamin B₁, cod-liver oil, copper, iron, zinc, and iodine (11). Synthetic mixtures of casein, lactose, cod-liver oil, yeast, and inorganic constituents, designed to resemble dried skimmed milk, are much less effective than the natural product. Milk seems to be well utilized even by very young fry (41). Dried whole milk as the sole article of diet for trout is not quite as good as dried skimmed milk.

The by-products of the sea-fishing industries are useful in feeding trout (25). Even the entire body of the herring affords an incomplete diet, however (40). The relative value of various animal organs for the growth of trout has been the subject of many investigations (8). Beef liver seems better than pork liver and better than spleen. Even fresh lungs are moderately satisfactory (40). Kidney and heart tissues are also quite complete in essentials (25).

FOOD CONVERSION

The efficiency with which fish are able to convert foodstuffs into body tissues has been the subject of many studies. Unfortunately many of these have been made without consideration of the water content or calorific value of the foodstuff, but solely in terms of the weight of food fed and the weight of fish produced. This renders it difficult to compare fish species with each other or with higher animals. In considering fish two factors need to be kept in mind, namely, that fish do not have to maintain a body temperature above that of their environment (8) and that species such as trout tend to grow at a slower rate than efficient converters of matter such as chickens, rats, and swine (9).

Trout compare very favorably with swine in their efficiency of conversion of foodstuffs. During considerable periods of growth they may build about a fifth of the meat consumed as foodstuff into body tissue (40). Over periods of sixteen to twenty-eight weeks it may require 2.7 to 5.3 units of dry food to produce a unit increase in body weight while for short periods these values may vary from 0.8 to 15.0 (26). These values show the fluctuations among groups of trout maintained under similar conditions. For four trout species the following values were obtained for the first forty weeks: lake trout (*Cristivomer namaycush*), 3.1; rainbow trout (*Salmo irideus*), 3.5; brook trout (*Salvelinus font.*), 4.7; and brown trout (*Salmo fario*), 5.1. From the fortieth to the ninety-second week all species improved in efficiency and values ranged from 2.3 to 2.8. From the ninety-second to the one hundred forty-fourth week the values were 2.9 to 3.8. Thus, throughout a period of nearly three years, lake trout consistently proved more efficient in the conversion of foodstuffs, while the other three species differed little after the first year (6).

Within certain limits the efficiency of conversion of foodstuffs is inversely proportional to the amount ingested (10). To secure the more efficient conversions it is usually necessary to decrease the growth rate in the case of trout. High protein diets that exceed 14 per cent tend to decrease this efficiency because they stimulate heat production that is of no use to the trout.

Pike differ as widely as trout in their conversion of foodstuffs, according to Scholz (57). His studies were made at temperatures of 17 to 19° C., where both growth and basal metabolism would be increased. Cornelius (72) summarized the literature and added some additional values from studies employing rainbow trout. At low levels of oxygen in the water he found inefficient conversion although he made no allowance for the movements of fish in the aquaria.

EFFECT OF TOXIC COMPOUNDS

The study of fish in their reactions to toxic compounds is usually undertaken by those interested in water pollution by industrial wastes or by those interested in the assay of drugs. It has long been known that fish eaters tend to excrete arsenic. Ellis (73) found that trout may contain considerable amounts of arsenic if it is found in a stream. He believes it is acquired by trout by eating the nymphs of the stone fly. Wiebe and coworkers (74) found that bass held in ponds treated with arsenic retained this element in their tissues. Sadolin (75) found

the arsenic to be present in some acid combination which was fat soluble.

Chlorinated water is toxic to goldfish if chlorine is present to the extent of 1 to 3 p.p.m. (76). Fluorine and its compounds have been given little attention. Strell (77) found hydrofluoric acid about twice as toxic as hydrochloric acid. Neff (78) found that fish teeth were mottled if there were fluoride in the water. The toxicity of such oxidizing compounds as hypochlorites, ozone, and peroxide has been studied by Hubbs (79). The dumping, daily, of more than two tons of tar acids and nearly a ton of cyanide into the Tees Estuary has led to many investigations concerning the effect of these compounds upon fish (80).

Herzfeld and coworkers (81) have employed fish for testing such compounds as thyroxine. The use of rotenone as an insecticide has stimulated the study of the toxicity of plant extracts for fish (82, 83). Drake & Busbey (84) have also studied the toxicity of organic thiocyanates. Marine gobies are rendered more sensitive to certain compounds like strychnine through preliminary treatment with 2,4-dinitrophenol (85). Temperature plays an important part in the susceptibility of fish to toxic compounds (86, 87).

Trout have been fed for long periods upon meats preserved with such compounds as alcohol, formaldehyde, and hypochlorite without injury unless an excess of the preservative is used (26, 31, 41). Long continued feeding of formaldehyde gives rise to special types of injury.

In the control of disease epidemics fish must often be subjected to drastic treatment. For brief periods fish can live in solutions of such oxidizing agents as potassium permanganate. Wolf (88) has studied the effects of solutions of this compound upon trout. In attempts to control some intestinal parasites trout have been fed such compounds as mercurous chloride, hexamethylenetetramine, carbon tetrachloride, beta naphthol, and resorcinol. Relatively large doses of mercurous chloride can be fed and this seems to be the best method for the control of *Octomitus salmonis* (26).

Amytal anesthesia has been studied with both fresh and salt water species (88). Dreyer (89) found that pilocarpine and atropine had no effect upon the gut of the skate and dogfish. The diffusion of alcohol into the tissues of live fish has been measured by Nicloux (90). The heart of the skate has been employed in studying the effects of adrenaline, histamine, atropine, and acetylcholine (98).

Fish have played little part in the advances of endocrinology with the possible exception of the revival of iodine therapy. Thymus feeding seems to stimulate the growth of fish (90, 91). Fish react to sex hormones and have been used as test animals (92, 93, 94, 95, 96). The red coloration of minnows has served as a test for Zondek's "intermedin" (97). Hess (99) has found that the islets of the pancreas of rainbow trout tend to degenerate as the result of overeating and lack of exercise. Binet and coworkers have used the expansion of the melanocytes of carp scales to test pregnant urine (100).

ENZYMES

The part played by enzymes in the physiology of fish has been studied with few species and under limited conditions. The classical problem concerning possible differences between the enzymes that function in poikilotherms and homeotherms continues to attract attention. Vonk (101) reviewed the problem and compared the pH optima for the pepsin of swine and pike. For swine it varied from 1.7 to 1.8 and for pike from 2.2 to 2.5. He observed the high concentration of pepsin in the stomach of the pike compared to that of the shark. Pyatnitzkii (102) concluded that the pepsin in the stomach of the frog was identical with that of higher animals. However, the trypsin found in the perch seems to differ from that found in the dog (103). Chesley (104) has studied the factors that influence the amylases in man compared to a fish species, menhaden. Chesley (105) has also compared the proteases, amylases, and lipases in several marine fishes. Gastric mucosa of perch has some peptic activity at 1° C., but is most active at about 30° (106). The digestive enzymes of the herring have been studied by Battle (107). The pepsin and trypsin increase in activity from 2.4° to 37.5° C. Battle finds that chitin is not digested. The acid gastric juice of the herring has the ability to digest the hyalodentine from the scales of fish that are eaten but not the fibrous layer. Babkin and coworkers (108) find that the fasting skate continuously secretes small amounts of very acid gastric juice and that this contains about 1 per cent of urea.

Lipases from the livers of carp and swine are inhibited to about the same extent by a series of alcohols (109). Beauvalet (110) finds that extracts of the anterior stomach of *Scyllium* hydrolyzes fibrin while the posterior portion provides enzymes that will split fats and starches in alkaline media.

In studying the structure of insulin and urease, Kawamoto (111)

has determined whether these and their degradation products will pass through the gills of carp. Normal and dialyzed insulin seem to exert both a hyper- and hypoglycemic function, when allowed to pass from the water through the gills. Urease fails to pass until it has been partly digested by trypsin.

Sammartino (112) finds the catalase of fish eggs is unchanged by fertilization. In rainbow trout this catalase increases during the first twelve days of development. It seems to be associated with the initiation of blood circulation.

BLOOD COMPOSITION

The properties of fish blood that have continued to attract interest are chiefly those concerned with the nitrogenous and the inorganic constituents. Vars (113) finds the blood of the pike and carp has about 59 mg. of non-protein nitrogen, 5 to 6 mg. of urea, and about 1 mg. of uric acid nitrogen per 100 cc. The fibrin may vary from 172 to 431 mg. per cent according to Zunz (114) who has compared the fibrin in fish blood with that of higher species. Demenier (115) finds great variability between species and season of the year in his determinations of the albumin and globulin of fish blood. Albumin ranged from 3.4 to 17.8 and globulin from 10.8 to 44.7 gm. per liter. Fontaine & Boucher-Firley (116), in their comparison of fresh and salt water species, found a range of 25 to 70 gm. per liter for the total protein of the serum, with large individual variations.

Fish form specific agglutinating sera of high titre when injected with living or dead bacteria (117). Toth (118) was unable to find any blood-group differences among carp. Among species he found that the agglutination properties of the sera varied widely but the cells were quite constant. The sera of shad proved very toxic when injected into such higher animals as mice and cats, due probably to the hemolysis that resulted (119).

The blood of the conger and murena has been analyzed by Boucher-Firley (120) who finds especially high values for serum protein in the murena. Tamura (121) investigated the lipids in the blood of some poikilotherms. The various forms of phosphorus in the blood of fish, eels, and turtles were determined by McCay (122). In goldfish, the glutathione of the blood was determined at three different temperatures by Binet & Weller (123). At 8 to 10° the total was 18.2 mg. per cent while at 28 to 30° it was 14.5 mg. per cent. During the spawning season Okamura (124) found high values for the

organic constituents in the blood of salmon. The water content of the blood of salmon during different stages of development was determined by Kuroda (125).

A series of new analyses of the blood of various fish species has been made in studies of the relation between the inorganic constituents and osmotic relationships (126, 127, 128, 129, 130, 131). Fontaine (132, 133) finds the inorganic phosphorus to be higher in the blood of teleosts than in that of selachians while there is little difference in the calcium. In studying the respiratory function of the blood Kokubo (134) finds that the pH of carp blood can be changed from 6.8 to 8.3 and the carbon dioxide from 5.7 to 68.4 vol. per cent. Resistance to change is more marked in carp than in *Leuciscus*. Root (135, 136) has found that the oxygen-combining power is related to the relative activity of the species under normal conditions.

GASEOUS METABOLISM

Acclimatization seems to play a part in the gaseous metabolism of fish. In the case of the mud sucker (*Gillichthys mirabilis*) Sumner & Wells (137) found that those individuals held at high temperatures and then changed to intermediate temperatures had different rates of metabolism from those held at low temperatures and brought to the intermediate. Those held at the higher levels and reduced to the intermediate had lower rates of metabolism than in the case of the reverse change. However, Washbourn (138) found that trout accustomed to swift water continued to use more oxygen than those used to sluggish water when both trout were held in slowly moving water. In his experiments the trout were anaesthetized with urethane.

The use of oxygen by embryonic trout has been studied by Wood (139). He finds this requirement to be a function of body weight rather than surface. The problem of the relation of body surface to metabolism in the case of fish remains unsolved. Wiebe (140) found that bass could tolerate very high levels of oxygen for brief periods. Adkins (141) has described a method for determining the basal metabolism of fish.

MUSCLE COMPOSITION

The proteins of haddock muscles have been split into three fractions by Logan (142), depending upon solubility in water, sodium chloride, and phosphate buffer. The press juice of haddock muscle

has also been studied by Schmidt-Nielsen & Stene (143). Flössner (144) isolated adenylic acid, adenine, hypoxanthine, and cytosine from fish muscles. The non-protein nitrogen in the muscles of skate, lobster, haddock, herring, and salmon have been compared by Campbell (145). Carp muscle has 0.35 per cent creatine and 0.02 per cent phosphocreatine, expressed as phosphorus, according to Palladin & Sigalova (146). Kernot & Speer (147) found the labile phosphorus for six fresh water fish species to vary between 8 and 26 mg. per 100 gm. They found another organic phosphorus compound more resistant to acid hydrolysis than phosphocreatine.

From the liver of codfish, Yoshimura & Nishida (148) have isolated tyrosine, leucine, serine, trimethylamine, hypoxanthine, histidine, methylguanine, and choline.

The end products of nitrogenous metabolism in fish are urea and trimethylamine from protein and urea and ammonia from purines (149). Pitts (150) noted the low urinary chloride in the urine of marine fish. Smith had already noted that marine species must excrete chlorides by some other channel than the kidneys (24).

In recent years the increased interest in the domestication of bass has stimulated a more intensive study of the many chemical factors that govern the nutrition of the lower organisms. Embody & Sadler (151) have given special attention to the fertilization of ponds for the intensive production of daphnia.

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CHEMICAL EMBRYOLOGY*

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CHEMISTRY AND PHYSICS OF THE NUCLEUS AND CHROMOSOME

Outstanding progress has been made in determining the distribution of certain components in the nucleus by Caspersson, Hammarsten & Hammarsten, and especially as continued by Caspersson. The absorption of ultraviolet light by nucleic acids is so great that less than 10^{-9} mg. is measurable from the absorption (using photography). The concentration of nucleic acid can be measured in a particle with diameter only four times the wavelength used. Enzymes are used to digest proteins in chromosomes, leaving nucleic acids *in situ*. The nucleic acid content of the nucleus varies greatly with the functional state of a gland cell. It alters during the mitotic cycle. During mitosis in living cells the nucleic acids localize in elements building the chromosomes.

In the giant salivary gland chromosomes of *Drosophila*, alternating light and dark bands (or discs), seen in material which has been fixed and stained, are found from cytogenetic evidence to correspond to the loci of particular genes. These bands are revealed in the living chromosomes by ultraviolet absorption in studies by Caspersson, and the chromosomes are shown to be made up of albuminous protein bearing intermittent discs or segments of very complicated structure which are rich in nucleic acid. The intervening segments are almost devoid of nucleic acid. Digestion of the protein causes the nucleic acid discs to fall freely apart. Some of these, in turn, may split into several thinner discs, not previously discernible as separate. This also correlates with the most recent genetic evidence.

Uber & Goodspeed, and Uber show by micro-incineration of pollen that heavier elements are localized in the chromosomes as compared with the nucleoplasm.

In a series of papers Wrinch continues an exceedingly interesting theoretical study of molecular structures which may be applicable to chromosomes, based on physical and protein chemistry and the requirements of cytology and genetics.

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GERM CELLS

Egg cells.—Many unfertilized eggs enter maturation upon being shed. Pasteels shows in a number of invertebrates that this is due to calcium (to some extent to potassium) in the new medium. Maturation is inhibited by narcotics, but not by anaërobiosis, potassium cyanide, or moniodoacetate. In the rabbit's egg Pincus & Enzmann (1) demonstrate that the first polar body forms if the ovarian egg is removed from the follicular environment. This is unaffected by pituitary hormones or thyroxine, and such eggs may be fertilized. Whitaker (3) finds that permeability of the unfertilized sea-urchin egg to water decreases greatly at maturation, corresponding to the increase when development is resumed at fertilization. Certain membrane alterations and the uptake of water in the trout's egg, which have commonly been referred to fertilization, are shown by Manery & Irving actually to proceed to the same extent in unfertilized eggs in contact with water. Goldforb (1) demonstrates that aging, unfertilized, sea-urchin eggs swell, and their properties approach those of fertilized eggs in that viscosity (2) and permeability to water (3) increase. Elster finds that aged eggs are more readily fertilized by foreign sperm.

The fertilizable life of unfertilized eggs is usually quite limited in spite of the large reserve of at least certain types of nutrients. Carter (1) finds that thyroxine extends this period in the sea-urchin egg, and reports (2) that it improves fertilization in unripe starfish eggs, in which respiration is stimulated. Whitaker (4) extends the period hundreds of per cent in *Urechis* eggs with 1 per cent dextrose or alcohol.

Holter examines the relations of the hydrolytic enzyme, peptidase, to the cytoplasmic granules in the unfertilized eggs of the sea urchin, *Arbacia*, and other invertebrates. Determinations on fragments of eggs stratified by the centrifuge, so that granules (including mitochondria, etc.) are segregated, show that peptidase activity is unrelated to the distribution of granules. Neither is it differentially distributed between the cortex and the cell interior (sand-dollar eggs). At least certain respiratory enzymes, on the other hand, appear to be associated with granules, since Shapiro finds in the unfertilized *Arbacia* egg that oxygen consumption by granular fragments (containing most of the yolk and echinochrome) is 88 per cent greater than by clear fragments.¹ The nucleus lies in the clear fragment and thus is

¹ The respiratory rates of the clear fragment and the normal egg are about the same, as is the sharp increase at fertilization. The respiratory rate of the granular (pigmented) fragment does not change at fertilization.

not the dominant site of oxidation. Results which correlate are those of Navez & Harvey, who show that the activity of indophenol oxidase is twice as great in the granular fragment.

Nucleic acids have been isolated from sea-urchin eggs by Blanchard. Potentials are considered by Dan and by Uspenskaja, and in the frog's egg by Hasama and by Dorfman. The latter correlates gradients of pH and rH with morphological polarity. In the hen's egg, Marza continues studies of the histochemistry of yolk formation, and with Chiosa investigates concentrations of potassium in regions of the developing egg. Osmotic pressure relations of the white and yolk are further investigated by Jolin and by Moran & Hale. Moran determines the bursting strength of the vitelline membrane. Romanoff & Grover follow the electrical conductivity of albumen, yolk, and fluids in several avian eggs during development.

Spermatozoa.—Carter (2) has stimulated unripe echinoderm sperm with thyroxine. Iwanow reports that mammalian sperm may remain motile in monohalogen acetic acid solutions indicating that glycolysis is not indispensable for performance of mechanical work. Machowka & Schegaloff find that in an electric field the sperm of a number of mammals migrate to both poles because of galvanotaxis superimposed on electrophoresis. Only negative charges are borne by the sperm. Rostand reports that frog sperm survive longer in 99 per cent heavy water than in distilled ordinary water, retaining fertilizing power without nuclear damage. Pincus & Enzmann (2) find that a heat-labile substance from rabbit sperm, like trypsin, causes dispersal of follicle cells surrounding the rabbit egg *in vitro*. They also show that sperm which have been irradiated with ultraviolet light initiate egg development *in vivo* although the sperm chromatin degenerates after penetration. Henshaw & Francis find that when sea-urchin (*Arbacia*) eggs are fertilized by sperm which have been x-rayed, cleavage is delayed, as when eggs alone are irradiated.

Fertilization.—Although, heretofore, blood has been found to inhibit fertilization, MacGinitie shows that eggs of the marine worm *Urechis*, when injected into the blood cavity of the male (in which sperm develop), are fertilized and grow rapidly to larvae. Kasovskaja fertilizes rabbit eggs *in vitro* with rat sperm (75 per cent dividing). Chase finds that temperature coefficients of rates of cortical reaction and membrane formation at fertilization decrease with rise in temperature, and in five echinoid eggs Moore determines the relative effectiveness of a series of cations in preventing the loss of

membrane-forming capacity in isosmotic urea. Clark, and Ancel & Vintemberger have studied the effects of chemicals on polyspermy in several invertebrates and in the frog.

Runnström (4) shows in the sea urchin that iodoacetate does not influence fertilization,² which leads him to conclude that not only glycolysis but also all activities involving free sulfhydryl groups are unessential for fertilization. The nature of developmental defects tends to support his view that carbohydrate breakdown is especially necessary for differentiation of the anterior region of the larva. Runnström (1) also finds that pyocyanine greatly increases the respiration of unfertilized and fertilized eggs (especially the former), and its effects in conjunction with hydrogen cyanide indicate that neither the iron-containing enzyme nor the dehydrase-substrate system limits the rate of oxidation. Örström finds a temporary burst of ammonia production at fertilization in the sea-urchin egg.³

In *Arbacia* eggs, Stewart & Jacobs show that permeability to ethylene glycol, diethylene glycol, and propylene glycol is approximately doubled at fertilization. Cole & Cole find that the electrostatic capacity of the plasma membrane of the unfertilized egg ($0.73 \mu\text{F}$ per sq. cm.) is practically independent of the frequency of the alternating current (10^3 to 10^7 cycles per sec.), while that of the fertilized egg varies inversely with the frequency (probably due to the fertilization membrane). Internal resistance is not changed at fertilization, being 4 to 6 times that of sea water, depending on frequency.

Numerous investigations in recent years indicate profound changes in the colloidal state of the egg following fertilization, presumably related to biochemical changes occurring at this time. An important advance is made by Mirsky who finds that about 12 per cent of the protein in sea-urchin eggs coagulates and becomes insoluble between three and ten minutes after fertilization. This coagulation, like that of myosin in contracted muscle, but unlike that caused by heat or acid, does not alter the sulfhydryl and disulfide groups, which suggests that it is due to dehydration of the protein particles. These may then come together to form a fibrous network. Mirsky considers the possibility that such a network might become a skeleton framework within which differentiation proceeds. Also see Harrison.

Artificial parthenogenesis.—In general, fish embryos have not

² See also Tyler & Schultz.

³ Acid is also produced [Runnström (3)].

developed far following artificial parthenogenesis, but Kasansky has reared feeding and growing young carp from unfertilized eggs activated by being placed in human saliva for three to five minutes. Pincus, and Pincus & Enzmann (2) extend the development *in vitro* of parthenogenetically activated rabbit eggs. Tschakhotine activates the Pholade egg, heretofore refractory to parthenogenetic agents, by point irradiation of the surface with a micro beam of ultraviolet light 5 μ wide.

Cell division (see also growth and differentiation).—Non-nucleated halves and quarters of *Arbacia* (sea-urchin) eggs are activated parthenogenetically by Harvey. Asters form and cell divisions in sequence may form blastulae with 500 cells which live for weeks. This is the greatest degree of development which has been attained in the absence of the nucleus.

Beams & King find that *Ascaris* eggs divide after being stratified in the ultracentrifuge at 400,000 G for an hour, and that when they are maintained in the centrifuge for more than four days under a centrifugal force of 150,000 G, cell division proceeds at the control rate. The protoplasm thus remains alive and functional⁴ under forces which Svedberg has used to separate proteins. This suggests that hyaloplasmic colloidal components are somehow bound together relatively strongly.

Clowes, Krah1 & Keltch show that dinitrocresol and other nitro compounds greatly stimulate the respiration of several species of eggs (to extents depending on the compound) while reversibly inhibiting cell division at stages of the mitotic cycle which are more or less species specific. Differences in the action of these compounds compared with the oxidation-reduction indicators are demonstrated and discussed by Krah1 & Clowes, and by Clowes & Krah1. Henshaw & Francis and Heilbrunn & Young study the cleavage-delaying effect of X-rays on *Arbacia* eggs, and the latter authors find that delay is augmented if ovarian tissue is present. Nelson & Brooks find that a photochemical effect reduces fertilizability and retards subsequent cleavage when unfertilized *Urechis* eggs are exposed to infrared light (0.7 to 2.0 μ).

EMBRYONIC GROWTH AND DIFFERENTIATION I

Tissue culture.—With plant-tissue extracts Bonner is able to culture and sub-culture parenchymous cells from the inside lining

⁴ Regarding metabolic effects, see page 478.

of bean pods, with cell division and tremendous cell elongation. As no essential change of tissue type takes place this is the closest approach to true tissue culture, as distinguished from regeneration, in plants (see also La Rue). Only the less elongated cells will establish new cultures, and so far this cannot be carried out indefinitely.

Gaillard finds that tissues, e.g., osteogenic cells, from chick embryos of different age require tissue juice from embryos also of different age for optimum growth *in vitro*. The effects on differentiation of the progressive changes in body fluids during ontogenesis are strikingly emphasized by his experiments on osteogenic cells *in vitro*. When these are cultivated successively in tissue juices from donors of ascending age (seven, ten, fifteen, eighteen days incubated, newly hatched, and adult hen), true bone differentiates. No such degree of differentiation is attained when juice from seven-day embryos is used successively throughout.

Ephrussi makes the interesting observation that certain tissues from hereditarily lethal mouse embryos proliferate normally *in vitro*, and differentiate. It thus appears that death of the lethal embryo is due to disturbed developmental correlations rather than to inherent properties of its cells. Hadorn (1) earlier found similar relations in tissues from lethal hybrid *Triton* meregones. Posterior parts of such lethals also develop well when grafted to normal *Triton* embryos (2).

Willis finds that embryonic cartilaginous primordia differentiate into specific bones when transplanted into brains of young rats.

Important advances in technique have extended tissue culture beyond the embryonic range. Carrel & Lindberg culture whole adult organs *in vitro* with sterile artificial medium pumped through the blood vessels, and Parker maintains adult (1) and embryonic (2) tissues for protracted periods in a state of functional survival, comparable to that in the adult body, instead of in a state of proliferation and growth.

Polarity and morphogenesis.—In *Fucus* the polarity and developmental pattern of the embryo [which is shown by Whitaker (2) to differentiate more rapidly in the light than in the dark] may be determined in dark-reared embryos by a brief exposure to unilateral light, most effectively many hours before differentiation actually takes place (Whitaker & Lowrance). Whitaker (1) shows that it may also be determined by establishing a pH gradient across the egg in sea water. The rhizoid forms, respectively, on the dark and on the acid side of the egg.

Lindahl reports that when sea-urchin eggs are stretched in passing through a capillary, the first end to emerge comes to be the ventral part of the embryo (if the axis of stretching does not coincide with the polar axis). The dorso-ventral axis is also determined by centrifuging.

Child & Watanabe and Watanabe show that when pieces of hydroid stem stained with methylene blue are subjected to anaërobiosis, patterns of rate of reduction of the dye coincide with subsequent regenerative morphogenesis. Regions which reduce most rapidly become hydranth ten to sixteen hours later. Reduction of the dye is reversible upon admission of air. Rulon finds similar relations in the living chick embryo using the oxidized form of Janus Green. Gradients of reduction rate not only correlate with visible developmental activity (and correspond to Hyman's earlier disintegration gradients) but, as in *Corymorpha*, in some cases (e.g., optic cup) a high reduction rate precedes morphogenesis. In these cases the metabolic differences cannot be attributed merely to different properties of tissues with already established morphological differences. High oxidizing activity is indicated in the processes underlying and preceding the morphogenesis. This is in keeping with Tyler's view (see below) that differentiation involves very appreciable energy requirements.

Energetics.—Tyler (2) finds that normally proportioned giant embryos from two fused sea-urchin eggs develop more rapidly than normal sized embryos. This extends his observations (1), previously reviewed by Needham,⁵ that normally proportioned half-sized embryos, while consuming oxygen at the same rate per unit mass, develop more slowly and therefore consume more oxygen to attain a given stage in differentiation. The present results strikingly agree with his interpretation of the earlier ones, from which these were predicted, namely, that energy available for form-changes in differentiation varies in proportion to the amount of material involved, but the energy required (per unit material involved) varies inversely as some function of the radius of curvature of the parts concerned; this is to be expected from physical theory. Thus while twice as much energy is available per unit time in a double-sized giant embryo, less than twice as much is required for form-change, which therefore proceeds more rapidly.

Tyler shows further in a number of marine invertebrate eggs that

⁵ *Ann. Rev. Biochem.*, 4, 452 (1935).

temperature coefficients of the rates of attainment of the various cleavage stages, and of later stages of differentiation, are approximately the same (3) and that the total amount of oxygen consumed at different temperatures to reach the same developmental stage is the same (4).⁶

In the sea-urchin egg Lindahl studies the effects of a number of ions and other agents some of which augment the animal and some the vegetal development of the embryo. In general, although with some exceptions and complications, agents which inhibit certain parts of the respiration tend to inhibit animalization and to favor vegetalization. This is true, for example, of lithium, and Runnström (2) shows (sand-dollar egg) that pyocyanine, which stimulates respiration, inhibits the endoderm-stimulating action of lithium. Runnström earlier found that inhibiting respiration augments lithium action. Lindahl has found that in the absence of calcium, sodium thiocyanate tends to animalize, if the eggs (sea-urchin) are treated before fertilization. Runnström & Thörnblom show that this effect is much increased in the presence of pyocyanine, which increases respiration.

Auxin (see also gene action).—The plant-growth substances known as auxins (including synthetic indolylacetic acid), in addition to being involved in plant-cell elongation and bud inhibition, have recently been shown by Thimann⁷ and Went and others to specifically stimulate or induce root differentiation (while inhibiting root elongation). They thus act specifically, depending on the cells, as growth hormones, as inhibitors, and as organ-forming substances. The remarkably clear-cut action of these substances in the development of a number of plants attaches interest to the manufacture of auxin at certain developmental stages in several animal embryos (Rose & Berrier; Robinson & Woodside). At present there is little evidence of any developmental function. Auxin may merely be formed as an incidental metabolite. The only report of favorable effect⁸ on animal growth is that of Martin du Pan & Ramsmeier who observe that preparations from orchid pollen accelerated cell division and neurulation in two salamander embryos. More cases, necessary to establish the point, will be looked forward to with interest.

⁶ Contrast Crescitelli, page 477.

⁷ Cf. *Ann. Rev. Biochem.*, 4, 545 (1935).

⁸ Exogastrulation in the sea urchin (Motomura) is probably not a specific effect since many inhibitory agents cause exogastrulation (see Waterman).

X-rays.—Skoog shows that hard X-rays, by means of oxidizing agents which they produce, destroy the plant-growth substance auxin in the plant without necessarily affecting the growth response to auxin or the mechanism of its transport. This appears to be the major factor in X-ray growth inhibition and the stimulation of lateral buds (which are inhibited by auxin). (Also see Chesley.)

The inhibiting effect of X-rays on limb-bud regeneration in larval salamanders appears to be due to local action (Butler), especially on the mesenchyme cells [Puckett (1)]. Differentiation is more inhibited than proliferation. Puckett (2) finds that differentiation is also more inhibited in hydroids. For further experiments on Amphibia, see Brunst & Chérémétiéva, and Chérémétiéva & Brunst.

Curtis, Cameron & Mills cause exogastrulation in Amphibia by exposing blastulae to X-rays (1,000 Roentgen units). The effect is not due to ozone. (Also see Waterman regarding sea urchins.)

EMBRYONIC GROWTH AND DIFFERENTIATION II

Insects.—Crescitelli finds that during pupation in the bee-moth the oxygen consumption and carbon dioxide production at first decline and then rise, while falling sharply again at the end. The R.Q. remains constant throughout at 0.69 over a wide temperature range. The total oxygen consumption during pupation is not the same at different temperatures, as previously found by others in several insects, but it is at a minimum at 30° C. (to which temperature the stock had been acclimated). Crescitelli & Taylor find that the concentration of reducing substances during pupation follows a somewhat similar curve, and give evidence to support the view that glucose is synthesized during pupation, probably from fat, which accords with the low R.Q. In *Drosophila*, Poulson shows that during pupation males consume more oxygen than females (see also Crescitelli), and Dobzhansky & Poulson find the usual pupal U curve for oxygen consumption, with protracted flattening in the trough. They consider the corresponding reorganization changes.

Hobson concludes that a fat-soluble factor necessary for the growth of blowfly larvae is a sterol. Cholesterol is especially effective. Smith finds that a growth-stimulating substance formed in fatigued frog's muscle, which causes blowfly larvae to grow to larger size, is thermolabile and passes from the contracting muscle into the frog's blood stream.

The developing grasshopper embryo provides unusual interest

because of the temporary arrest of development in the diapause. During developing stages respiration is partly stable and partly sensitive to cyanide. In diapause the respiration is low and it is the cyanide-sensitive respiration which is absent (see Needham).⁹ Bodine & Boell (4) show that methylene blue does not elevate the respiration of developing stages (unless it has been first suppressed by cyanide) but that it elevates the respiration in diapause approximately to the rate in development. It thus can substitute for the respiratory mechanism which is blocked either by cyanide or naturally at diapause. In this connection it is interesting that alteration of the microcellular structure by ultracentrifuging with forces up to 400,000 G, so that the cell components are stratified, greatly depresses the respiration of developing stages but has no effect in the diapause [Bodine & Boell (5)]. Centrifuging thus has an effect similar to that of cyanide. The respiration of ultracentrifuged *Ascaris* eggs (Huff & Boell) is reduced to about a quarter (without affecting cleavage rate) and this is cyanide stable, while much of the respiration of the non-centrifuged eggs is cyanide sensitive. Here also the effect of centrifuging is primarily on the cyanide-sensitive respiration. In the grasshopper, Boell shows that the R.Q. starts near unity and declines to very low levels as development progresses, which supports Needham's view of a sequence of energy sources during development. Conversion of fat to carbohydrate is indicated at certain stages.

Studies are reported of the activity, during development, of catalase (Williams), peroxidase [Bodine & Boell (1)], tyrosinase [Bodine & Boell (2, 3)], and indophenol oxidase [Bodine & Boell (6)]. The activity of the latter fails to correlate with the respiratory rate of the embryo.

Amphibia.—Wolsky, Tazelaar & Huxley report differential acceleration of growth of certain parts of the frog embryo by pilocarpine, hydrochloric acid, and other agents, so that larval proportions are altered. Twitty shows that a substance in *Triturus* embryos which paralyzes *Amblystoma* embryos without affecting their growth acts specifically on the nervous system of the *Amblystoma*. This was demonstrated by creating embryos with the muscles of one species innervated with nerves of the other. Wills finds no sex difference in the respiration of *Triturus* embryos.

Waddington, Needham & Brachet find in *Triton* and *Rana* that

⁹ *Ann. Rev. Biochem.*, 4, 456 (1935).

dorsal lip tissue (organization center) consumes no more oxygen (per unit weight) than ventral ectoderm. Since Brachet (1) found greater carbon dioxide production, a difference in type of metabolism is suggested; this is found by Brachet (2) in *Discoglossus* in which oxygen, carbon dioxide, and fixed acids were measured simultaneously. The R.Q. of the dorsal lip material is 1.1, compared with 0.77 for ventral ectoderm. A greater rate of oxygen consumption is also found in the dorsal lip tissue in this form. It may be that the change in metabolic type at the dorsal lip is the forerunner of a change which spreads over the embryo since in the frog embryo Brachet & Needham (2) find that the type of metabolism shifts markedly at gastrulation. The R.Q. is elevated and aerobic glycolysis increases. Glycogen is now utilized for the first time. Glycogen which is combined with protein is used in preference to free glycogen, which is not used at all until neurulation. In agreement see Takamatsu. Concerning anaerobic metabolism see Latinik-Vetulani.

Distribution of glycogen in the amphibian embryo is determined by Heatley, and by Tanaka. During development, determinations have been made of free and combined phosphorus (Zieliński), of a number of enzymes (Takahashi; Yamasaki), and of urea and uric acid (Takamatsu & Kamachi).

Chick.—Brachet & Needham (1) find that arginase activity in the developing chick embryo correlates with the growth rate (also see Baldwin). Gregory, Asmundson & Goss report that glutathione concentration correlates with the rate of cell proliferation when two breeds of chickens with different rates of proliferation are compared. Mystkowski finds that cathepsin activity is very low throughout development in the chick embryo and no effects could be detected on protein synthesis *in vitro*.

ORGANIZER PHENOMENA

Fischer, Wehmeier, Lehmann, Jühling & Hultsch report that a number of fatty acids induce amphibian medullary plate formation as do acid fractions of tissue extract which lose this power when neutralized. They believe that a large variety of substances induce (evocate) by virtue of acidity and suggest that acids present may explain the activity of unsaponifiable ethereal fractions of tissue extracts. That this is not necessarily so is convincingly shown by Waddington, Needham, Nowiński, Lemberg & Cohen who find high activity in such fractions after neutralization. These authors give

further evidence that sterol-like substances in crude glycogen (impurities) and in brain extracts from which cephalin is removed are the inductive (evocating) agents in these preparations. The work of Brachet (2) and of Waddington, Needham & Brachet suggests that the organization center is a region of carbohydrate metabolism (see also page 479), and the latter authors show that living ventral ectoderm subjected to methylene blue, which presumably stimulates carbohydrate metabolism,¹⁰ acquires the property of inducing (evocating) neural tube. Dürken shows in *Triton* that the inductive property of dorsal lip tissue may be destroyed with ultraviolet light without killing the tissue or preventing gastrulation.

A leading question which is brought into focus but is not yet answered, and which, from the nature of the case, will be very difficult to answer, is whether a large variety of substances are neural inductors (evocators) or whether this property is confined to a specific substance, or group of related substances, widely present in tissues but ordinarily inactivated by combination with other substances. The actions of the wide variety of activating agents (e.g., heat, acid, etc.) in this case would consist in dissociating the combination (e.g., by denaturing protein). If the latter is the case the work of Waddington, Needham, and coworkers (see Needham,¹¹ including effects of oestrogenic hydrocarbons) points quite convincingly at the present time to sterol-like substances commonly inactivated by combination with protein and perhaps glycogen. A penetrating review is provided by Waddington, Needham & Brachet and excellent reviews are also given by Woerdeman and, especially in relation to the field concept, by Weiss.

CHEMICAL AND HORMONAL DETERMINATION OF SEX

In the marine worm *Bonellia*, Baltzer showed long ago that high percentages of larvae become females in normal sea water but if they settle down on the proboscis of a female they parasitize it and develop into males which come to reside in the intestine and uterus of the female. Many larvae become males or intersexes in sea water containing a piece of proboscis (Baltzer). Water extracts of proboscis or intestine cause male formation (Baltzer; Nowiński) while

¹⁰ This is not specifically proven in the present instance and the authors agree that the methylene blue may be exerting its effect in one of several other possible ways.

¹¹ *Ann. Rev. Biochem.*, 4, 454 (1935).

acetone extracts do not (Nowiński). Water extracts are effective after boiling, acting at high or low pH (Baltzer), which suggests a hormone-like substance. However, other agents as well cause male formation, notably acid [Herbst (1), Heydenreich]; copper [Herbst (1)], although this metal is not present in the proboscis (Mutscheller); magnesium [Herbst (3)]; and potassium in sufficient amount [Herbst (2)]. Herbst attributes the effects of potassium and magnesium to regulation of water uptake.

Ökland shows that the injection of female hormones into the hen does not affect the sex of the offspring, and Dantchakoff (4) reports that sex hormones applied to the larvae do not influence sex in *Drosophila*.

Pincus & Kirsch find that when female rabbits after copulation are injected daily with theelin (oestrone) or theelol (oestriol) the early cleavage rates of the embryo are unaffected (as also *in vitro* in the presence of the hormones) but the embryos disintegrate in the blastocyst stage.

Some years ago Frank R. Lillie made observations suggesting that when twins of cattle start as a male and a female the earlier differentiation of the gonads of the male partly suppresses the development of the gonads and gonoducts of the female which then becomes a sterile intersex (free-martin). More recent attempts by several workers failed to obtain this type of hormonal relationship when gonad primordia were grafted on to the chorio-allantoic membrane of the chick embryo. However, the direct application of sex hormones to early chick embryos is now shown to have profound effects upon the sexual development (see below).

The action of female hormones on the chick.—Willier, Gallagher & Koch have injected theelin and theelol (0.5 to 2.0 mg.) in ethylene glycol into the albumen of 350 chick eggs at forty-eight hours of incubation and dissected on the nineteenth or twentieth day. The eggs were from brown leghorns and also from crosses in which the embryos carried sex-linked plumage characteristics directly identifying the genetic sex. Depending on the dosage, genetic males are converted into all degrees of intersexuality including forms almost indistinguishable from normal females. The left testis which is normally hermaphroditic in its earlier structure develops differentially to assume the shape and structure of an ovary. In extreme cases the right testis is suppressed and shows ovarian tendencies. Essentially the same results are obtained by Wolff & Ginglinger (1) by placing 25 to

3000 U.I. of folliculin in oil or water on the chorio-allantoic membrane at about five days' incubation. The effectiveness is much reduced at eight days when the gonads are more differentiated, and absent at twelve days. Genetic males, strongly converted to the female type, tend to resume male characteristics weeks or months after hatching (2). Dantchakoff reports similar conversions of genetic males into intersexes with folliculin injected into the allantoic sac (1), with decreasing effectiveness after the fourth day (2). She also finds that feminized males ultimately revert toward maleness (3) although often retaining ovules in gonads (5) or even containing eggs and albumen in the oviducts after thirteen months (6).

Willier, Gallagher & Koch find that theelin and theelol do not affect Wolffian ducts of either sex but large doses cause enormous swelling of the oviducts in both sexes. Wolff & Ginglinger (1), and Dantchakoff (6) also find that the oviducts are enlarged by folliculin.

The action of male hormones on the chick.—Wolff (1) shows that synthetic androsterone (0.35 to 1.0 mg.) converts females into intersexes and (2) also converts males into intersexes. It thus exhibits mild feminizing as well as strong masculinizing action. Oestrogenic action is found when androsterone is injected into castrated female mice [Wolff & Ginglinger (3)]. Willier, Gallagher & Koch find that extracts from bull testis (10 to 200 bird units) have no effect on chick gonads of either sex. Male hormone extract from human urine, however, whether or not female hormone is present, as indicated by the rat vaginal smear test, has no effect on the gonads of the females (although it inhibits the oviduct). It causes the Wolffian ducts in both sexes to swell tremendously and converts the left testis of the male into an ovotestis. This feminizing activity of the male hormone extract is attributed to the presence of androsterone and dehydro-androsterone. As in the case of female hormones the action on the genital ducts is duct-specific and independent of the genetic sex.

GENE ACTION DURING DEVELOPMENT

A very satisfactory analysis of the action of a single gene difference is seen in the work of van Overbeek on a dwarf strain of corn compared with its normal heterozygote. Van Overbeek shows that the dwarfed growth is due to a low content of plant-growth hormone (auxin). This is due to a higher rate of auxin destruction in the tissues rather than to lack of production. The catalase activity is

found to be correspondingly higher, compared with the normal, and peroxidase activity also is high. Catalase activity in young hereditarily normal plants was almost doubled by exposure to high temperature for an hour. Auxin destruction increased and the dwarfed type of growth followed. The type of growth of the dwarf strain thus appears to result from oxidative destruction of growth substance due to changed oxidation-reduction properties of the cells.

In flies the imaginal disc primordia of a number of adult organs, which do not differentiate until during pupation, are present in the larvae as discrete bodies. Ephrussi & Beadle, and Beadle & Ephrussi (1, 2) have developed an effective pipette technique for transplanting these primordia into larvae of different mutants and species of *Drosophila*. This line of work is very promising since it combines embryology and genetics in so favorable a material as *Drosophila*. Ovarian primordia differentiate and become functional in another species, as the genital ducts of the host often connect to the transplant, and progeny are produced after mating. The most important results concern the developmental interactions of the genetic constitutions of the graft and the host. Light is thrown on the time as well as the nature of the actions of particular genes. Progress has been made especially in analyzing the determination of eye pigment in a large number of eye-color mutant strains. Thus the eye disc of a genetically vermilion-eyed fly, transplanted into the larva of a genetically wild (red-eyed) host, develops into an eye in the abdomen of the adult host, but the eye is red because of the effect of diffusible factors in the red-eyed host. On the other hand, a red-eye disc in a vermilion host, being itself provided with the factors, becomes a red eye unless it also bears the form factors for Bar or unless the donor is considerably younger than the host. A similar relation holds between the cinnabar eye mutant and the red-eyed wild type, but it is further found that a vermilion-eye disc in a cinnabar host becomes a red (wild type) eye. Exploring relations among a large number of eye-color mutants in this fashion Beadle & Ephrussi (2) find it possible to explain certain data by supposing that, of three substances concerned in determining the final pigment, the first is a precursor to the second which in turn is a precursor to the third. None, or one, or two, or three of these substances may form, depending on the genes present, which may also affect the rates of transformation, and all of these factors affect the final eye color.

The hypothetical substances referred to above are not to be con-

fused with the pigment itself which appears at a later stage. Schultz has analyzed the eye pigments of many strains of *Drosophila*, especially by means of the absorption spectrum. There are two chemically related pigments, red and yellow. The various eye colors depend on the absolute and relative concentrations of these two pigments as well as upon other factors such as the size of the pigment granules. Concerning elaboration of the pigments during development and also broader aspects of the relations between genes and development, see Schultz.

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SCHOOL OF BIOLOGICAL SCIENCES
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PLANT PIGMENTS*

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It is impossible to treat adequately in this review all phases of advance made during the past two years in the chemistry of the plant pigments. For this reason the reviewer has limited the discussion largely to the advances made in the determination of the chemical structures of the carotenoid pigments and the chlorophylls. Only a few observations concerning the chemistry of other pigments which seem to have special significance are included.

CAROTENOID PIGMENTS

β -Carotene.—In the last review of Plant Pigments, Kuhn (1) stated that "Karrer's formula for β -carotene has been definitely established by means of degradation studies with chromic acid." Since that statement was made Kuhn & Brockmann (2) have presented a rigorous proof of the correctness of Karrer's formula. The name assigned is 1,18-bis [2,2,6-trimethylcyclohexen (6)-yl]-3,7,12,16-tetramethyloctadecanonaene.

γ -Carotene.—The marsh dodder, *Cuscuta salina*, has been found to contain relatively large quantities of γ -carotene (3).

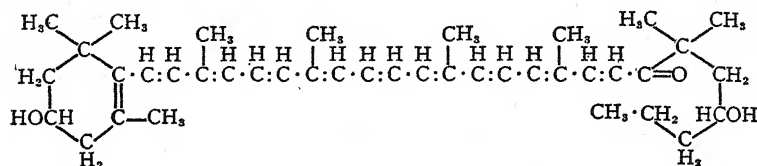
Capsanthin.—This pigment has been isolated from the Perfection pimiento and has been identified with the pigment obtained from Hungarian paprika (4). Capsanthin is optically active. It contains two hydroxyl groups before and three hydroxyl groups after complete hydrogenation. It possesses ten conjugated carbon-to-carbon double bonds and one keto group which must be a part of the conjugated double-bond system, as judged by its unreactive nature and by the absorption spectrum of capsanthin. Oxidation produces 4.4 mols of acetic acid, α,α -dimethyl succinic acid, and α,α -dimethyl malonic acid, but no acetone.

A series of stepwise oxidations carried out on capsanthin has yielded an array of compounds which are analogous to those obtained from β -carotene under like conditions (5). The following compounds have been obtained: capsanthinone, $C_{40}H_{58}O_5$; anhydrocapsanthinone, $C_{40}H_{56}O_4$; capsanthylol, $C_{30}H_{42}O_3$; capsylaldehyde, $C_{27}H_{38}O_3$; and 4-oxy- β -carotenonealdehyde, $C_{27}H_{36}O_4$.

* Received January 20, 1937.

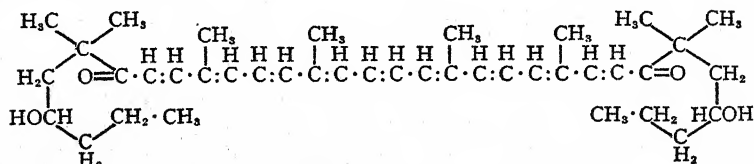
Capsanthin has been reduced to capsanthol, $C_{40}H_{60}O_8$, with aluminum isopropylate (6). This reduction product possesses absorption maxima which are more distinct than the maxima of capsanthin and displaced 35 m μ farther to the violet, thus indicating the reduction of a carbonyl group at the end of a conjugated chain. Reaction with alkali in the absence of air causes a rearrangement in the molecule, the nature of which is unknown.

The structure proposed on the basis of these facts is given in formula I (5, 7).



I. Capsanthin, $C_{40}H_{58}O_8$

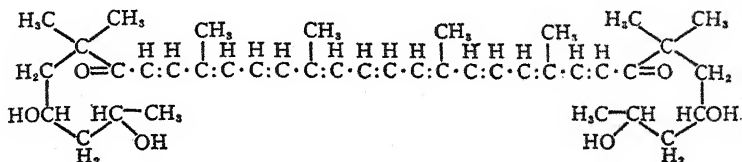
Capsorubin.—In nature capsorubin is closely associated with capsanthin (7). Capsorubin possesses nine double bonds (9.65, 9.58, 9.31), two hydroxyl groups, four methyl groups (4.24, 4.13), and no isopropylidene groups. The two remaining oxygen atoms are assumed to have ketonic functions because of the similarity of the absorption spectrum of capsorubin to that of bixindialdehyde and the marked difference between the positions of the absorption maxima in alcohol and in benzene. These observations lead to formula II for capsorubin.



II. Capsorubin, $C_{40}H_{60}O_4$

Fucoxanthin.—Experiments have been reported which bear on both the chemistry and physiology of fucoxanthin (8). The new work indicates an empirical formula of $C_{40}H_{60}O_6$ [compare $C_{40}H_{56}O_6$ (Karrer)]. Fucoxanthin has been found to be optically inactive, contrary to previous work. It melts at 166 to 168° C. and shows absorption maxima in carbon bisulfide at 510, 477 m μ . When fucoxanthin is exhaustively hydrogenated it loses four oxygen atoms and yields a perhydro compound of the empirical formula $C_{40}H_{78}O_2$. A prelimi-

nary structural formula (III) based on insufficient evidence has been proposed (8).



III. Fucoxanthin, $C_{40}H_{60}O_6$

One disturbing feature of this structure is that the observed absorption maxima lie at very much shorter wavelengths than would be predicted from the conjugated system proposed (cf. capsorubin).

Violaxanthin.—Recent experiments on the hydrogenation of violaxanthin, $C_{40}H_{56}O_4$, have given a hydrogenation equivalent of 10 as compared to the former value of 11 (9). The new value is more in keeping with the positions of the absorption maxima.

Zeaxanthin.—Measurements have shown that zeaxanthin obtained from leaves is optically active $[\alpha]^{18}_{6678} = -41^\circ$ [chloroform (10)]. This agrees with the commonly accepted formula for zeaxanthin, which contains two asymmetric carbon atoms.

A very interesting problem in carotenoid chemistry is the transformation of one pigment into another. One such conversion, which is of especial significance, in that one naturally occurring pigment has been transformed into another, is the conversion of rhodoxanthin into zeaxanthin by reduction (11).

New carotenoid pigments.—Many new carotenoid pigments have been isolated from plants during the past two years. The increasing rate at which these pigments have been discovered is due largely to the ease of isolation, purification, and identification which the newer methods of adsorption (12, 13), absorption (14, 15, 16, 17), and microchemical analyses (18, 19) have brought.

A list of recently described plant carotenoids is given in Table I.

The discovery of lycoxanthin and lycophyll, which are assumed to be mono- and dihydroxy-derivatives of lycopene, is of particular interest (20). Their occurrence points to the existence of a whole series of compounds which bear the same relationship to lycopene that the xanthophylls bear to carotene. A further variation in the structure of lycopene, namely the shift in the positions of the double bonds, is illustrated in the structure (formula IV) proposed for rhodoviolascin (21).

TABLE I
NEW CAROTENOID PIGMENTS AND THEIR PROPERTIES

Pigment	Source	Formula	Melting Point C. ^o	Specific Rotation 18 (α) _D ²⁰	Sol- vents†	Absorption Maxima Wavelengths in m μ	Carbon Double Bonds	Function of Oxygens	Reference
Antheroxanthin	<i>Lilium tigrinum</i>	C ₄₀ H ₆₈ O ₃	211*		1 2	512.5, 481, 448 490.5, 460.5, 428			(24)
Lycophyll	<i>Solanum Dulcamara</i>	C ₄₀ H ₆₆ O ₂	179		1 3	546, 506, 472 504, 473, 444		2 OH	(20)
Lycoxanthin	<i>Solanum Dulcamara</i>	C ₄₀ H ₅₆ O	168*		1 3	547, 507, 473, (443) 503, 472, 443		1 OH	(20)
Capsorubin	<i>Capsicum annuum</i>	C ₄₀ H ₆₀ O ₄	201		1 3	541.5, 503, 468 506, 474, 444	9.5	2 OH 2 C=O	(7)
Euglenarhodon	Euglena	C ₄₀ H ₄₈ O ₄	227-228*		1	520		4 C=O	(25)
Rhodoviolascin	<i>Rhodovibrio</i> bacteria <i>Thiocystis</i> bacteria	C ₄₂ H ₆₀ O ₂	218*	Benzene† O	1 4	573.5, 534, 496 526, 491, (465)	13	2 CH ₃ O	(22, 21)
Rhodopin	<i>Rhodovibrio</i> bacteria		168		1 5	547, 508, 478 501, 470, 440		1 OH	(21, 26)
Rhodopurpurin	<i>Rhodovibrio</i> bacteria		162		1 5	550, 511, 479 502, 472			(26)

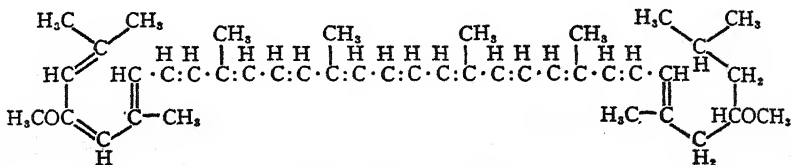
Petaloxanthin	Flower of <i>Cucurbita Pepo</i>	$C_{40}H_{58}O_3$	211-212*		1 4	514.5, 481 483, 451.5		(27)
Spirilloxanthin	<i>Spirillum rubrum</i>	$C_{48}H_{60}O_3$	218-219*		1 4	566, 530, 501, 417 528, 495, 469	15	(23)
Myxoxanthin	<i>Rivularia nitida</i>		117-118*		1	489		(28)
Isolutein	Green leaves	$C_{42}H_{60}O_4$		$CHCl_3$ O	4	473, 446		(10)
Neoxanthin	Green leaves	$C_{40}H_{56}O_4$		$CHCl_3$ +32	4	466, 437		(10)
Flavoxanthin-c	Green leaves	$C_{40}H_{56}O_3$ or O_4		$CHCl_3$ -56	4	450, 424		(10)
Flavoxanthin-b	Green leaves	$C_{40}H_{56}O_3$ or O_4		$CHCl_3$ +75	4	450, 424		(10)
Violaxanthin-b	Green leaves	$C_{40}H_{56}O_4$		$CHCl_3$ +33	4	471, 442		(10)
Citraurin	Orange		144-145		1 3	523, 488, 457 486.5, 457		(29)

* Melting point corrected.

† Wavelength of light and temperature not recorded.

‡ Solvents: (1) carbon bisulfide; (2) chloroform; (3) benzene; (4) ethanol; (5) petroleum ether.

Carotenoids in purple bacteria.—The principal carotenoid pigments in the purple bacterium, *Rhodovibrio*, are rhodoviolascin and rhodopin. Rhodoviolascin, $C_{42}H_{60}O_2$, contains two methoxyl groups, no more than one isopropylidene group, and thirteen double bonds all of which are probably conjugated, as judged from absorption-spectrum measurements. This is the first carotenoid pigment in which methoxyl groups have been found.



IV. Rhodoviolascin, $C_{42}H_{60}O_2$

Rhodopin has not been isolated in quantities sufficient for thorough analysis. What analytical data have been obtained indicate the presence of one hydroxyl group and twelve double bonds.

The nature of the relationship that exists between the two pigments rhodoviolascin obtained from *Rhodovibrio* (21, 22) and spirilloxanthin from *Spirillum rubrum* (23) has not been determined. Absorption measurements indicate a close relationship.

Physiological investigations.—Many attempts have been made to determine the physiological significance of the carotenoid pigments in plants but so far they have given little if any definite information.

One method of attack has been to determine the ratios of the different pigments present in a variety of plant materials and in the same type of plant material under widely different conditions. While no correlation of these ratios with the physiological functions of these pigments has been made from this work, the factual basis for such correlations has been increased. From such surveys it is apparent that (a) the ratio of chlorophyll to carotene does not vary greatly in barley seedlings in different states of chlorosis (30); (b) β -carotene is always present in green leaves in relatively large amounts and is often accompanied by α -carotene (13, 31); (c) leaves contain not less than twelve xanthophylls of which lutein is the major constituent and is always accompanied by zeaxanthin (32); (d) in the handling of plant materials in different ways after harvesting, the proportions of the different pigments are changed (8, 10, 33); (e) the yellowing in pumpkin flowers is accompanied by very considerable shifts in the ratios of different carotenoid pigments (27); (f) each group of algae

possesses characteristic carotenoids (34); and (*g*) such decidedly different photosynthetic organisms as purple bacteria and green leaves both contain a pigment complex consisting of green pigments accompanied by a series of carotenoid pigments (23, 26, 35).

In other investigations attempts have been made to show that the carotenoids play a definite part in the photo-responses of plants. The striking similarity between the absorption curves of the carotenoid pigments (α - and β -carotenes, lutein, and zeaxanthin) and the curve relating phototropic response of oat coleoptiles to wavelength of the incident light (36) has led to the assumption that this response is due to the absorption of light by the carotenoid pigments. This supposition has received support from the demonstration of carotenoid pigments in these coleoptiles (37). The presence of the carotenoids in all the photosynthetic organs and organisms thus far investigated has led to the belief that these pigments may play a rôle in photosynthesis. Recently it has been asserted that fucoxanthin aids in the photosynthetic reactions of various algae (38). The evidence is insufficient, however, to be considered as proof. On the other hand, it has been demonstrated that photosynthesis in the purple bacteria is actually decreased by the presence of carotenoids (39), due probably to their absorption of light. In considering photosynthetic yields in relation to the light absorbed by the pigments, it is highly important to know the spatial arrangement of the pigments in the photosynthetic organ (40), since the fraction of the light absorbed by each pigment depends on this physical condition (41).

To carotene has also been ascribed the physiological function of a plant hormone, active in the production of new rootlets (42).

During the last two years three researches have dealt with the problem of the chemical transformations of the carotenoid pigments in plants. The reactions investigated were as follows: the influence of various factors on the production of lycopene in tomatoes (43, 44); the formation of β -ionone from carotene by the alga *Trentepohlia iolithus* (45); and the rapid and almost complete disappearance of carotenoids caused by grinding etiolated seedlings (10). It will be of interest in the future to see to what physiological processes these transformations are due, especially to determine whether or not they are caused by specific enzymes.

Physico-chemical investigations.—A very important series of papers has appeared (46) dealing with the spectroscopy of compounds containing conjugated double bonds. The absorption spectrum of ly-

possesses characteristic carotenoids (34); and (g) such decidedly different photosynthetic organisms as purple bacteria and green leaves both contain a pigment complex consisting of green pigments accompanied by a series of carotenoid pigments (23, 26, 35).

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copene at low temperatures (-196°C.) is much sharper and contains many more distinct absorption bands than at room temperature. An analysis of the absorption spectrum has indicated that a differentiation can be made between double bonds inside and outside of the conjugated system.

It is of interest that the porphyrin spectra possess the fundamental polyene spectra overlaid with other characteristic frequencies (46).

Quantitative absorption curves have been obtained for many carotenoid compounds (2, 15, 16, 47). Where comparative values are available the agreement in many instances is excellent. Whatever discrepancies exist are as yet inexplicable.

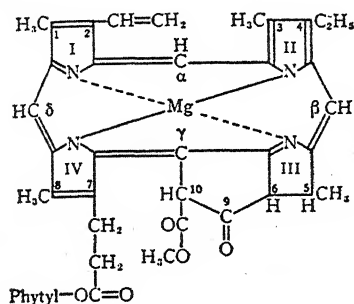
The positions of the long wavelength absorption maxima of certain carotenoids determined by means of a spectroscope supplemented with a copper-ammonium filter have been found to lie farther toward the red than when they are determined with a spectrophotometer. The promiscuous reporting of the maxima determined by these two methods has caused considerable uncertainty as to what the true values are. The causes for this discrepancy have been investigated recently. The values obtained with the spectrophotometer have been found to be the same regardless of the conditions used. The values obtained with the spectroscope and filter have been found to vary with the conditions used and have been shown to be the consequence of physiological optical contrast effects (17) combined with effects of visibility, the relative emission intensities of the light source at different wavelengths and the absorption of the copper-ammonium filter, all of which are superimposed on the absorption of the pigment (16). In view of these observations the values determined with the spectrophotometer are to be preferred.

A significant relation between the viscosities of solutions of carotenoid pigments and the structures of the pigments has been found (48). The results obtained confirm, to an extraordinary degree, those predicted.

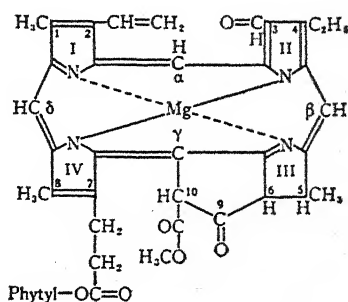
CHLOROPHYLL PIGMENTS

A structural formula for chlorophyll-*a* was shown in the last review of plant pigments (1). That formula has been altered in the following respects: (1) the ethylidene group in position 2 has been replaced by a vinyl group; (2) the pyrrol ring III has been changed to a pyrrolin ring; and (3) the double bonds have been assigned other positions. The new structure is represented by formula V (49).

Furthermore, a structural formula (VI) for chlorophyll-*b* has been proposed (50).



V. Chlorophyll-*a* (49)



VI. Chlorophyll-*b* (50)

Chlorophyll-a.—Evidence for the presence of a vinyl group in chlorophyll-*a* derivatives has been gained in two ways, by the hydrogenation of chlorophyll compounds and by oxidation of their dihydro derivatives.

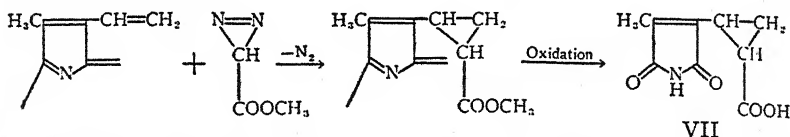
When chlorophyll-*a* and ethyl chlorophyllid-*a* are hydrogenated catalytically in neutral solution, they absorb only one mol of hydrogen. The dihydro compounds are still colored, and absorb light at only slightly shorter wavelengths than do the unhydrogenated compounds. The fundamental type of absorption is changed but little.

Oxidation of the chlorophyll-*a* derivatives, such as phaeophorbid-*a*, chlorin-*e*₆, and chlorin-*e*₄, yields only half as much methylethylmaleicimide as oxidation of the dihydro derivatives (49). This is well explained by the structure (formula V). From such a structure the production of methylethylmaleicimide would be expected only from ring II before hydrogenation, and from both rings I and II after hydrogenation. This agrees with the facts mentioned.

Diazoacetic ester adds specifically to double bonds in the side chains of porphyrin compounds. The existence of a double bond in the side chain of chlorophyll-*a* has been definitely established through the addition of this reagent to chlorophyll-*a* derivatives (phaeophorbid-*a*, pyropheophorbid-*a*, chlorin-*e*₆-trimethyl ester, and chlorin-*e*₄-dimethyl ester) (51). The failure of diazoacetic ester to react with dihydrophaeophorbid-*a*, phaeoporphyrin-*a*₅, phylloerythrin and oxophylloerythrin (51) has substantiated this conclusion.

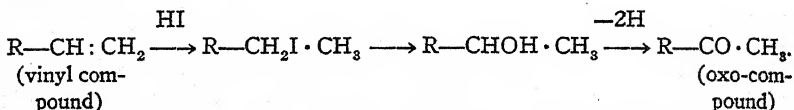
The addition compound of diazoacetic ester with pyropheophorbid-*a*, when oxidized, yields an acid of the structure represented by

formula VII. Inasmuch as the addition reaction takes place in the manner shown, there can be little doubt as to the existence in chlorophyll-*a* of a pyrrole ring substituted with a methyl and a vinyl group in the β -positions:

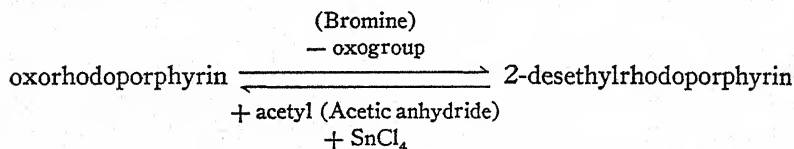


If chlorophyll-*a* contains an ethylidene group instead of a vinyl group, as formerly proposed, a much greater change in the absorption of the addition compounds with diazoacetic ester would be anticipated than that which is actually observed (51).

When certain chlorophyll-*a* derivatives are treated in air with hydrogen iodide dissolved in glacial acetic acid, the "oxo-reaction" occurs. By this reaction a keto group is produced as is shown by the formation of an oxime and by reduction to a secondary alcohol (51). The following equations represent the mechanism assumed for the reaction:



The "oxo-group" has been identified as an acetyl group by synthesis. The following reaction is an example of such a synthesis (53):



The synthetic acetyl compounds have been fully identified with the original oxo-derivatives (52, 53).

Some compounds in the chlorophyll-*a* series that are known to contain the vinyl group, notably phaeopurpurin-7, phaeopurpurin-18, and chlorin-*p*₆, fail to give the oxo-reaction. This failure may be ascribed to the tendency of these compounds to react in other ways, chiefly to form porphyrins (53).

The optical activity of chlorophyll-*a* and -*b*, and of many of their derivatives, was attributed to the asymmetry of carbon atom 10. When, however, pyropheophorbide-*a* was found to be optically active, even though C₁₀ was no longer asymmetric, it was realized that another

asymmetric center must be present. In consequence a number of compounds were tested for optical activity and it was found that the phorbids, chlorins, and purpurins are all optically active while the porphyrins are optically inactive (54).

Since it is known that the change from the phorbids and chlorins to the porphyrins, by means of hydrogen iodide, is accompanied by a saturation of the vinyl group, without change in elementary composition, it has been assumed that this change is an isomerization in which two hydrogen atoms are transferred to the vinyl group from some other place in the molecule (51). Because optically active compounds become inactive through this transfer (54) it is logical to suppose that the original site of either or both hydrogens is asymmetric and that by the removal of these hydrogens the asymmetry is destroyed.

Asymmetry can be caused by placing the two hydrogen atoms on two carbons in any of several positions but only two of these, C₅ and C₆, appear to be likely.

It is highly probable that C₅ is asymmetric and the site for one of the hydrogens, because pyrrochlorin, isochlorin-*e*₄ and phyllochlorin, in which C₅ is apparently the only asymmetric center, are all optically active.

The evidence that C₆ is asymmetric and the seat of the second hydrogen is based on the isomerism of chlorin-*p*₈ and pseudochlorin-*p*₈ (55). Chlorin-*p*₈ easily forms an inner acid anhydride while the pseudochlorin-*p*₈ does not. This is explained by a *cis-trans* relation of the H and COOH on C₆. While this is a plausible explanation the fact should be mentioned that the porphyrins from these chlorins show the same propensities for anhydridization.

The oxidation of phaeophorbid-*a* to 10-acetoxy-vinyl-phaeoporphyrin-*a*₃-monomethyl ester with silver oxide in glacial acetic acid has demonstrated the formation of a porphyrin without isomerization (56). This dehydrogenation has been followed quantitatively and has demonstrated that two hydrogen atoms are responsible for the difference between phorbid and porphyrin and the probable cause of the optical activity of the chlorophyll-*a* derivatives. This supports the proposal that ring III is a pyrrolin ring.

The most logical positions for the two extra hydrogen atoms are C₅ and C₆ for the following reasons: (a) the difference in the stability of the isocyclic ring in the phorbids and in the porphyrins (54); (b) the difficulty encountered in the isomerization of the chlorins to the porphyrins by treatment with hydrogen iodide when C₆ is substi-

tuted only with hydrogen (57); (c) the increase in activity of the enolizable hydrogen on C_{10} in the porphyrin oximes in comparison with the corresponding phorbid oximes (58); (d) the small change produced in the absorption of light by the chlorins when the carbomethoxy group is removed from dihydrochlorin- e_4 -dimethyl ester to form dihydrophytylchlorin-monomethyl ester (59).

Heretofore the positions of the methyl and phytyl alcohol groups in chlorophyll-*a* have been uncertain. Now it has been proved that the methyl is in the carbomethoxy group attached at C_{10} , by demonstrating that it is lost when phaeophorbid-*a* is transformed into pyropheophorbid-*a*. As a corollary, the phytyl must be placed in the propionic acid group attached at C_7 . The methyl group is native to the original pigment as is shown by the preparation of phaeophorbid-*a* by the use of solvents free of methyl alcohol (49).

In previous structures proposed for chlorophyll-*a*, the double bonds have been assigned to more or less arbitrary positions. Although they cannot be placed definitely even now (perhaps they never can be fixed definitely due to the resonance of the porphyrin structure), they can be given more rational positions on the basis of spectroscopic observations.

If a double bond were placed between C_7 and ring III it would cause undue strain in the porphyrin structure; therefore the alternate position between C_7 and ring IV has been chosen (54). The double bonds attached to rings I and III have been given analogous positions because of spectroscopic evidence obtained through substitution of carbonyl groups in rings I, II, and III (60). The small change in absorption caused by the removal of a carbomethoxy group from C_6 in the chlorins indicates that C_6 is not bound by a double bond in these compounds (59). That the vinyl group is conjugated with the double bond system of the phorbin nucleus is evidenced by the change in absorption caused by its saturation.

Taking account of these facts, along with many others gained from spectroscopic observations, the system of double bonds shown in V and VI has been proposed.

Exception has been taken to this type of crossed conjugation because of the stable aromatic nature of the porphyrin nucleus. A completely conjugated system has been offered instead (61).

So far the magnesium atom cannot be given a definite berth in the chlorophyll-*a* molecule. Spectroscopic evidence shows that its introduction into the phorbin nucleus does not alter the spectral type (62)

but only changes the relative intensities of the absorption bands, increasing particularly the absorption of the red band (59, 63). On the other hand the introduction of magnesium into the porphyrin nucleus causes a change in spectral type (64). These observations have not yet been interpreted in terms of structure.

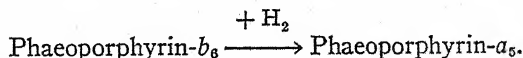
The introduction of magnesium into porphyrin compounds causes only a slight change in their atomic spacings, molecular volumes, and solubilities; therefore the metal is assumed to take a central position. In order to account for the relatively small change in polarization induced by the magnesium, the two ionic bonds holding it have been directed diagonally across the porphyrin nucleus (61). Because of the stabilization in the phorbin nucleus brought about by the magnesium, a completely co-ordinate binding of the metal has also been proposed (65).

The number of active hydrogens observed in the magnesium-free chlorophyll derivatives is always one less than in the magnesium-containing compounds (58). If the magnesium has replaced the two imide hydrogens, a difference of two active hydrogens would be expected. As yet this remains unexplained.

The reactivity of the molecule is considerably altered by the presence of magnesium. The introduction of magnesium increases the hygroscopicity, the tendency to allomerization, and the stability to decarboxylation and to hydrogenation (57, 65).

Chlorophyll-b.—The structure of chlorophyll-*b* is represented in formula VI. It differs from chlorophyll-*a* only in the replacement of the methyl group by a formyl group, $\text{HC}=\text{O}$, on C_3 . Such differences in properties and reactions as exist between the two chlorophylls can be explained by this difference in structure (50).

The close relationship between chlorophyll-*b* and -*a* has been demonstrated by the conversion of chlorophyll-*b* derivatives into chlorophyll-*a* derivatives (66) by catalytic hydrogenation:



By this reaction the formyl group has been reduced to the methyl group.

The presence of the following groups in chlorophyll-*b* have been demonstrated:

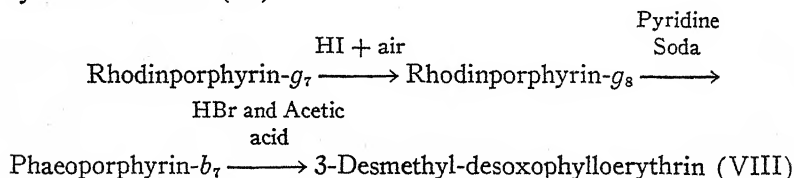
1. A vinyl group; by the formation of dihydrophaeophorbide-*b* (49) and by the addition of diazoacetic ester (50, 51, 66).
2. Two carbonyl groups; by formation of a dioxime (66).

3. A formyl group; by addition of malonic ester to rhodin-*g*-trimethyl ester (67), by addition of hydrogen cyanide to rhodin-*g* (50), by oxidation to a carboxyl group in rhodin-*g* [iron salt (50)].
4. Magnesium; by removal with acid and reintroduction with Grignard reagent (68).
5. Phorbin nucleus; by synthesis and comparison with analytical material (69).

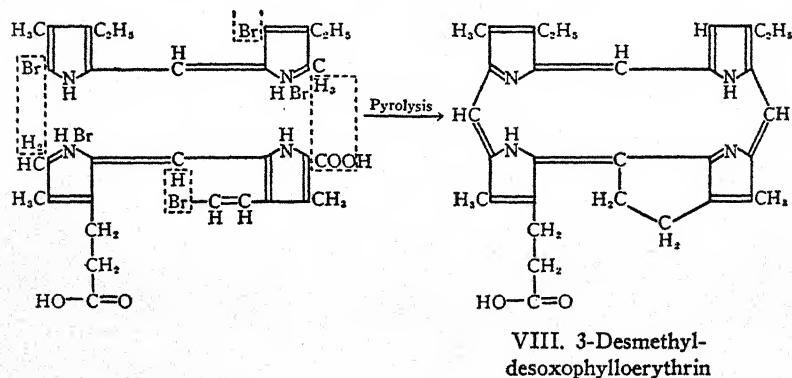
The structures of rings I to IV can be demonstrated in much the same way as they were in chlorophyll-*a*.

The structure of ring I is shown by oxidation of phaeophorbid-*b* and rhodin-*g* and their dihydro derivatives because methylethylmaleic imide is produced only after the vinyl group is reduced (49). Oxidation of the addition compound of rhodin-*g*-trimethyl ester with diazoacetic ester yields the same acid (VII) as was obtained from the corresponding addition compounds in the *a*-series.

The reduction of phaeoporphyrin-*b*₈ to phaeoporphyrin-*a*₈ indicates the correct formulation of ring II. This structure is further established by oxidation of the H—C=O group to a carboxyl group, removal of the carboxyl group and identifying this compound with synthetic material (67).



3-Desmethyl-desoxophylloerythrin (VIII) has been synthesized and fully identified with this analytical material (69).



The sequence of groups and the structure of the phorbins nucleus in chlorophyll-*b* have been established by this synthesis as well as the position of the formyl group.

Ring III, formula VI, should yield citraconic imide on vigorous oxidation and decarboxylation. This acid has been obtained from rhodin-*g* and neorhodinporphyrin-*g*₈ (49).

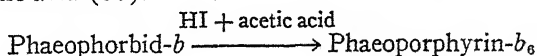
The presence of the propionic acid group on C₇ and the ring system IV has been settled by the formation of hemopyrrolcarboxylic acid by the reduction of rhodinporphyrin-*g*₇ with hydrogen iodide (67) and the formation of hematic acid by the oxidation of rhodinporphyrin-*g*₈ (49).

Chlorophyll-*b* and its near derivatives, phaeophorbid-*b*, the rhodins and *b*-purpurins, are optically active (50). It is assumed, for reasons similar to those already given for chlorophyll-*a*, that the seat of asymmetry lies in the pyrrolin ring III (49, 50).

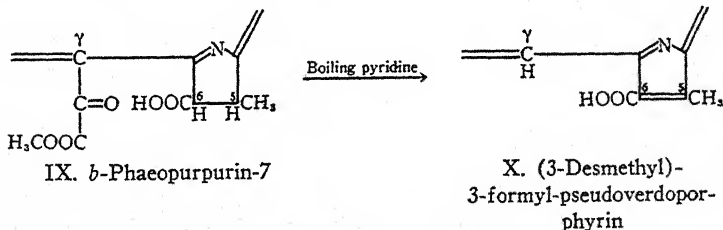
In chlorophyll-*b* the phytyl and methyl alcohol groups are given the same positions as in chlorophyll-*a* and for the same reason (49).

The disposition of the double bonds and the attachment of the magnesium follows from analogy with chlorophyll-*a*.

As in the chlorophyll-*a* series, porphyrins may be produced in the *b*-series by treating chlorophyll-*b* derivatives with hydrogen iodide in glacial acetic acid (66).

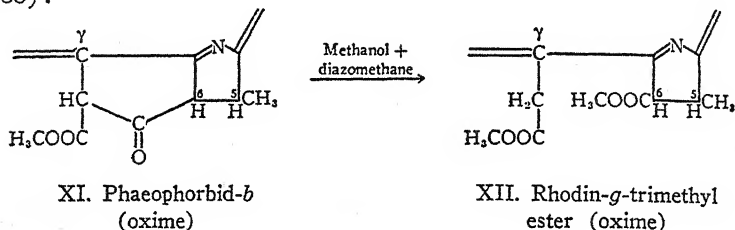


Here also the hydrogen atoms are transferred from the pyrrolin ring III to the 2-vinyl group. Vinyl porphyrins can be obtained by removing hydrogen atoms from C₅ and C₆ by oxidation, without simultaneously reducing the 2-vinyl group or affecting the 3-formyl group (50):

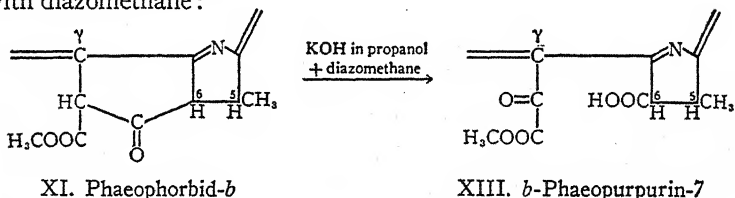


The transformations of the isocyclic ring are analogous to those in the *a*-series. To insure yields comparable to the *a*-series it has often been necessary to protect the formyl group by forming the oxime.

Methanolysis of the isocyclic ring in phaeophorbid-*a* produces chlorine-*e*. The analogous reaction in the *b*-series produces rhodin-*g* (66):



Purpurins are also formed in the *b*-series (50) by treating phaeophorbid-*b* first with propyl alcoholic potassium hydroxide and then with diazomethane:



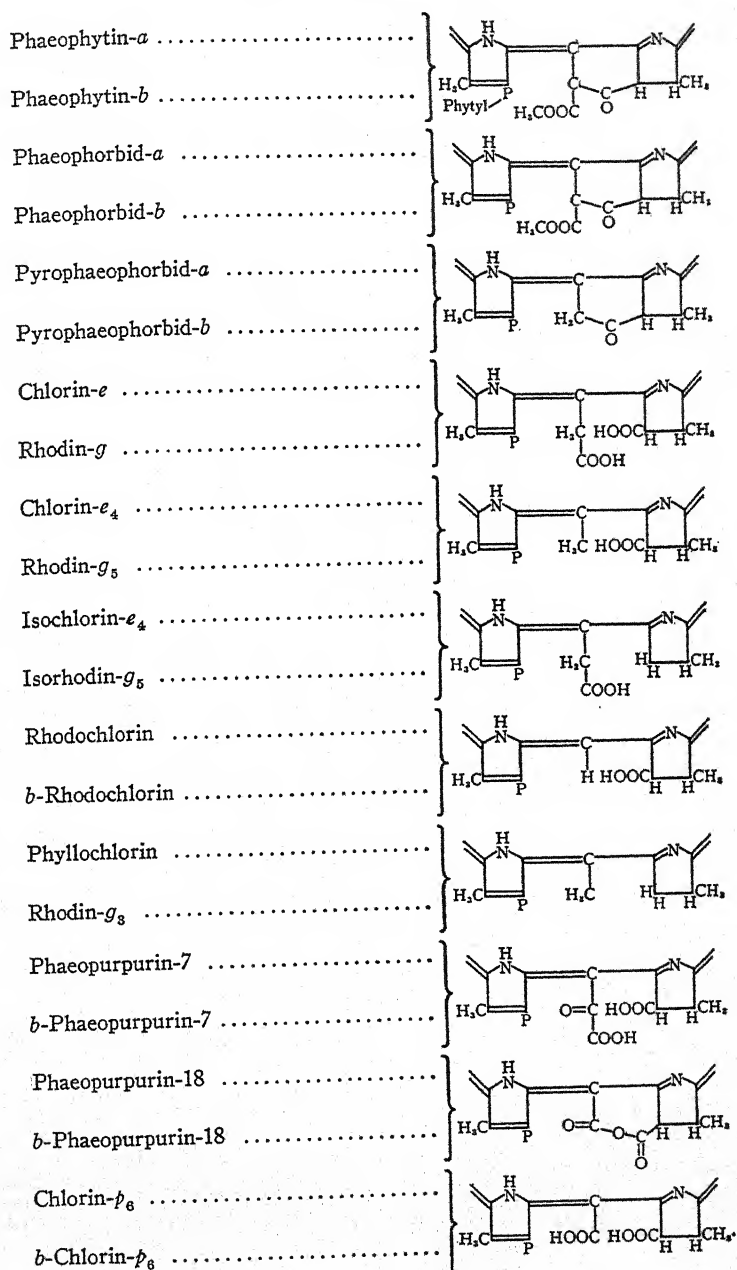
In the purpurin reaction an intermediate product is found in the *b*-series (metastable rhodin) which is much more stable than the intermediate metastable chlorin formed in the *a*-series.

A whole series of decarboxylations of the rhodins and *b*-purpurins have been carried out. The products formed have furnished valuable material for optical measurements. From these measurements, optical rotation and absorption spectra, evidence for the existence of pyrrolin ring III and the arrangement of the double bond in chlorophyll-*b* has been obtained (50).

The hydrogen atom on C₁₀ is especially labile in both chlorophyll-*a* and -*b*. It is the enolization of this hydrogen which is assumed to be responsible for the phase test. The ready oxidation of C₁₀ either by air or quinone (allomerization) eliminates the phase test in both chlorophyll-*a* and -*b* and gives rise to a series of compounds which have played an important part in these structural investigations (56, 66, 68).

A table of structures¹ on the opposite page shows the relation

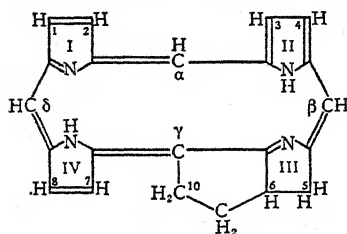
¹ The portions of the structures omitted are the same throughout for each series and may be supplied from formulae V and VI. "P" symbolizes the propionic acid group —CH₂ · CH₂ · COOH.



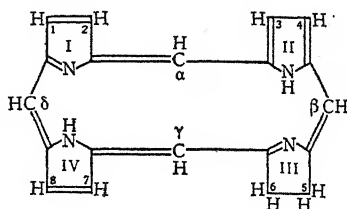
between the derivatives of chlorophyll-*a* and the corresponding derivatives of chlorophyll-*b*.

The synthesis of porphin has been accomplished during the past two years (70, 71). This substance is of particular interest because it is the typical compound in the porphyrin series.

Nomenclature.—Both chlorophyll-*a* and -*b* can be derived formally from the same nucleus. It has been proposed that the typical compound be called Phorbin (XIV) and the corresponding compound without an isocyclic ring be called Chlorin (XV) (62).



XIV. Phorbin



XV. Chlorin

It has also been proposed that the prefix "meso-" be substituted for "dihydro-" where dihydro- designates a saturated 2-vinyl group and that "vinyl-" be prefixed to the names of porphyrins containing a 2-vinyl group in place of the usual 2-ethyl group.

Biological.—Recent experiments have made it evident that the enzyme chlorophyllase is relatively specific. This enzyme hydrolyzed the synthetic phytyl, geranyl, and cetyl esters of phaeophorbide-*a* but not the *l*-menthyl or *d*-bornyl esters. Neither were the di-*l*-menthyl nor the di-cetyl esters of mesoporphyrin hydrolyzed (72).

Apparently an equilibrium is reached when about 43 per cent of the phaeophytin has been hydrolyzed.

The rôle which the two chlorophylls play in photosynthesis has not yet been determined. It is very instructive, however, that photosynthetic organisms have been discovered in which no chlorophyll-*b* could be found. The three algae for which this holds true are *Porphyra tenera* (market preparation called Asacusa-Nori), *Bangia fuscopurpurea*, and *Polysiphonia nigrescens* (49).

Many biochemical and physiological problems concerning chlorophyll in living organisms have been discussed in several short reviews on the development of chlorophyll (73, 74, 75), the inheritance of chlorophyll (76), and the state of chlorophyll in the photosynthetic organ (77, 78).

Bacteriochlorophyll.—The discovery of photosynthetic activity in the purple bacteria has stimulated investigation of the chemical nature of the green pigments in these organisms. Previous work (79) has shown that these pigments are closely related in structure to the leaf chlorophylls. Recent experiments have revealed the close relation of bacteriochlorophyll-*a* (the more abundant of the bacterial green pigments) to chlorophyll-*a* (80). This close relation is shown by the change in the absorption spectrum of bacteriomethylphaeophorbid-*a* caused by oxime formation, namely the shift of the absorption spectrum to shorter wavelengths without alteration in type. This is contrary to what would be expected if this pigment were similar to chlorophyll-*b*.

Only a small quantity of the second green pigment, the "b-component" is present in the extracted pigments.

The green pigments from *Rhodobacillus palustris*, *Rhodovibrio*, and *Thiocystis violacea* are identical as near as can be determined (80).

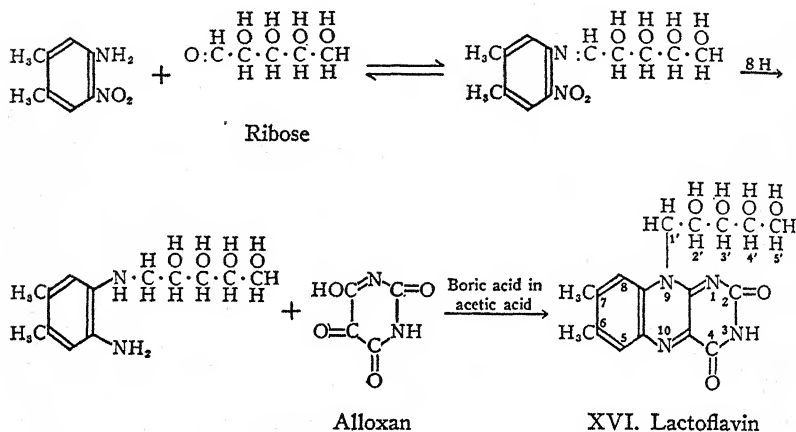
Bacteriochlorophyll-*a* when treated with acid yields bacteriopheophytin and, in the presence of methanol, bacteriomethylphaeophorbid-*a*, $C_{36}H_{38}N_4O_6 \pm 2H$ (79, 80). Chlorophyllase hydrolyzes bacteriopheophytin-*a* to bacteriopheophorbid-*a* (50), which on methanolysis forms bacteriochlorin trimethyl ester (80). Treatment of bacteriopheophorbid-*a* with hydrogen iodide gives a compound identical in all respects (including mixed melting point) with oxopheophorphyrin-*a*-ester, although the conditions for the oxo reaction were not used. The presence of the oxo group in the resulting compound has been taken as evidence for the existence of this group in the natural pigment (80). Bacteriopheophorbid is dehydrogenated by silver oxide to the porphyrin (56). From this evidence bacteriopheophorbid-*a* has been assigned a structure like phaeophorbid-*a* except that an acetyl group has been substituted for the 2-vinyl group in the latter compound.

Other observations that do not agree with such a formulation are: bacteriomethylphaeophorbid contains only two active hydrogens instead of the expected three (58); no optically active derivatives of bacteriochlorophyll have as yet been identified; and bacteriochlorophyll differs so greatly from chlorophyllid-*a* in spectroscopic properties (81).

OTHER PIGMENTS

Lactoflavin.—The structure of lactoflavin (vitamin B₂) has been established by synthesis as 6,7-dimethyl-9-*d*-ribitylisoalloxazine. It

was synthesized in the laboratories of Karrer and of Kuhn simultaneously (82, 83). The simplest synthesis (84) which appears to give good yields, based on the difficultly obtainable ribose, is given by the following reactions:



In nature, the pigment, lactoflavin-5'-phosphoric acid, is attached to a protein carrier. This complex is the yellow oxidation ferment of yeast. The lactoflavin-5'-phosphoric acid has also been synthesized by a series of several steps (85) and has been added to a protein carrier to form the synthetic enzyme. *In vitro* the synthetic enzyme acts as an oxidation catalyst just as does the natural product (86).

By measuring the dissociation of the complex formed between protein and the three pigments: 3-methyl-lactoflavin, lactoflavin, and lactoflavin-5'-phosphoric acid, it has been shown that both the free imide and the phosphoric acid groups combine with the protein (87). This is the first time that experimental proof of the specific nature of the union between protein and pigment has been obtained.

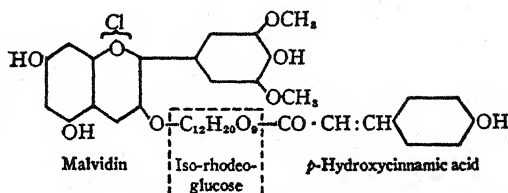
Combination with protein makes the oxidation-reduction potential of the pigment more positive, increasing its potential at pH 7 from -0.181 (0°C.) to -0.06 volt (0°C.) (88).

The photochemical reactions of lactoflavin have indicated that the groups attached to nitrogen atom 9 are photochemically activated. When lactoflavin is photolyzed, the ribityl group is split so as to leave a methyl group attached to nitrogen atom 9 (lumilactoflavin). Other flavin pigments with groups attached at nitrogen atom 9, which contain a primary alcohol group, are oxidized photochemically to the

corresponding acids either in the presence of air or of another molecule of pigment (89).

Anthocyanins.—The adsorption method, used so successfully in the separation and purification of the carotenoid pigments, has been adapted to the purification of the anthocyanin pigments. By this sensitive method impurities have been detected in supposedly pure compounds. The two anthocyanins from peony, cyanin and paeonin, which differ only in a methoxyl group, have been successfully separated and purified on an aluminum oxide column (90).

Two new anthocyanins, negreteine and tuberin, have been obtained from the violet potato. The structure of tuberin is not yet established. Negreteine has the structure represented by formula XVII.



XVII. Negreteine chloride

This pigment is noteworthy because it has been reported to contain a new biose, isorhodeose-glucoside (91).

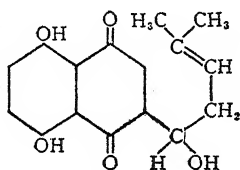
Flavanones.—Although the flavanones contain a potentially asymmetric carbon atom, only two, matteucinol and desmethoxymatteucinol, have been isolated in the optically active state. These pigments are easily racemized and it is probable that the reason why other members of this class have not been obtained in their optically active state is that they are racemized during their isolation. The racemic mixture of the two flavanones has been separated into optical antipodes by forming the *l*-menthoxy-acetyl esters (92). The method will be applied to other members of this class to determine whether the pigments isolated are racemic mixtures.

Alkannin and shikonin.—The structure of alkannin, the red anthraquinone pigment in the root of *Alkanna tinctoria*, has been reinvestigated and a new structure proposed (XVIII). This compound has been found to be the optical antipode of the pigment, shikonin, from the roots of *Lithospermum erythrorhizon*. "In alkannin and shikonin are found for the first time both of the optically active forms

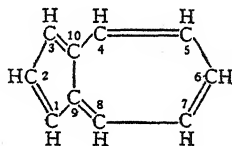
of a plant pigment occurring in nature, the levorotatory form as alkannin and the dextrorotatory form as shikonin." The racemic compound formed from these pigments also occurs in nature; to it the name shikalkin has been given (93). When hydrogenated, the hydroxyl group in the side chain is removed (cf. fucoxanthin). The 3-isohexyl-naphthazarin so formed, alkannan, accompanies alkannin in nature.

Azulenes.—A new azulene, lactarazulene, has been isolated from the fungus, *Lactarius deliciosus*, L. (94).

The chemical structures of the azulenes (the blue hydrocarbons found in many ethereal oils) have been discussed by Pfau & Plattner (95) who have proposed the following typical structure (XIX) for these compounds.



XVIII. Alkannin



XIX. Azulene

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THE ALKALOIDS*

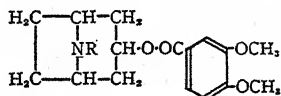
BY ERNST SPÄTH

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Papers published before the biennium 1935–1936 are discussed if necessary for an understanding of new work, or if they were omitted from earlier reviews. No attempt is made to be exhaustive. Unfinished research is recorded if the results obtained seem to be of sufficient interest. Collaborators are seldom mentioned in the text. Their names, however, will be found in the bibliography.

A good example of the fruitfulness of search for new alkaloids is a paper published by Orekhov (1) who, among 400 specimens of Russian poisonous plants that had not yet been chemically investigated, detected sixty-seven new genera containing alkaloids. An additional ten plant families, besides fifty-seven known already, have been found which yield alkaloids. Orekhov gives a list of several alkaloids which occur in more than one family. Späth (2) has listed those of which more than one optical isomeride has been found in Nature.

Alkamines.—During recent years several new alkamine esters have been examined. The best known alkaloids of *Convolvulus pseudocantabricus* are convolvine and convolamine (3). The former may be hydrolysed to veratric acid and nortropine; convolamine proved to be the N-methyl derivative (Ia) of convolvine (Ib). Both possess good local anaesthetic properties.



Ia. Convolamine (R = CH₃)

Ib. Convolvine (R = H)

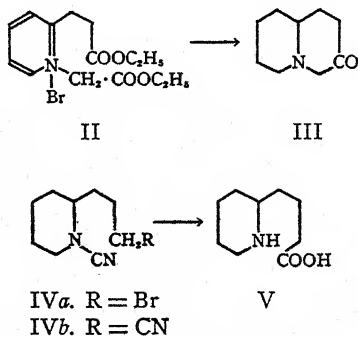
Orekhov (4) hydrolysed platyphylline (from *Senecio platyphyllus*) into platynecine, C₈H₁₅O₂N, and platynecic acid. Menschikov (5c) obtained lactic acid, trichodesmidine (C₈H₁₃O₂N), and methyl-isobutyl-ketone from trichodesmine; the ketone was formed by loss of carbon dioxide from a ketonic acid. Fission of lasiocarpine (5b) and heliotrine (5a), both from *Heliotropium lasiocarpum*, gave another alkamine, heliotridine (C₈H₁₃O₂N). The acid cleavage products were

* Received February 17, 1937.

angelic acid and lasiocarpic acid ($C_8H_{16}O_5$) from lasiocarpine, and heliotric acid ($C_8H_{16}O_4$) from heliotrine. A further alkamine, $C_8H_{13}O_2N$, is retronecine, which Manske (6) and Barger (7) obtained when different *Senecio* alkaloids, retrorsine, senecionine, and squalidine, were saponified. These alkaloids yielded isomeric acids ($C_{10}H_{14}O_4$) which were termed retronecic, senecic, and squalinecic acids respectively. There is little progress to report in the investigation of these acids. The Russian authors (4a, 5), however, succeeded in showing that their alkamines, platyphylline, trichodesmidine, and heliotridine, possess the same skeleton, by reducing them to a tertiary saturated base, $C_8H_{15}N$, termed heliotridane. From heliotridane, which is bicyclic, Menschikov has obtained (8a), by exhaustive methylation, hydrogenation, and dehydrogenation, a pyrrole base. Synthetic experiments (8b, c) have not yet enabled him to establish the constitution of heliotridane.

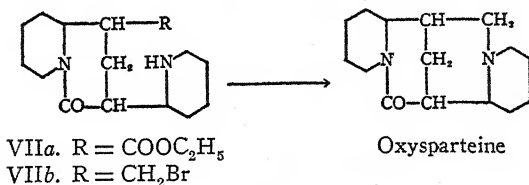
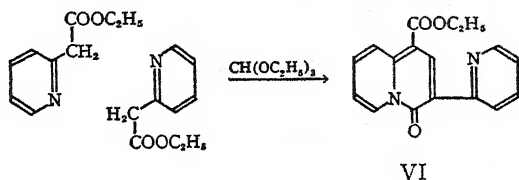
Lupinane-sparteine group.—Clemo has effected two new syntheses of *norlupinane* which are strictly confirmatory of the structure assigned to this important binuclear system:

Ethyl-pyridinium-1-acetate-2- β -propionate bromide (II) was reduced (9a) to ethyl-piperidyl-1-acetate-2- β -propionate; this was condensed by means of powdered potassium to 3-keto-octahydropyridocoline (III); in a similar way, 2-keto-octahydropyridocoline was obtained (9b), and both were reduced to furnish *norlupinane* (octahydropyridocoline).

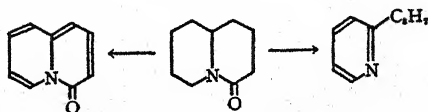


A ring enlargement of indolizidine (octahydropyrrocoline) was described by Ochiai (10) who treated the cyanogen-bromide degradation product, IV a, with potassium cyanide. The product, IV b, was then hydrolysed to the amino acid, V, which was converted easily into α -*norlupinone*. Rydon (11) synthesised α -methyl- α' -*n*-amyl-

glutarimide, which had not been fully identified before; it is one of the most important degradation products of anagryne.¹ Kondo (12) has given an interesting synopsis of the genetic relations between some alkaloids derived from *norlupinane*, i.e., the alkaloids of some Papilionatae, and aphylline and aphyllidine (from *Anabasis aphylla*, Chenopodiaceae). Winterfeld (13), who formerly claimed to have obtained 2-methylpyrrolidine from sparteine, now points out that the fourth ring is not a methylpyrrolidine nucleus but is a six-membered one. This is in agreement with Clemo's synthesis of *dl*-oxysparteine (14): Two mols of ethyl-pyridyl-2-acetate were condensed with $\text{CH}(\text{OC}_2\text{H}_5)_3$ to furnish VI, which on catalytic reduction yielded

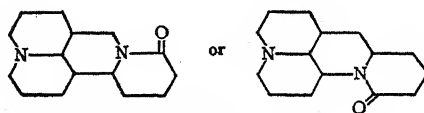


VII *a*. After a Bouveault reduction, the -OH group was replaced by bromine; the product, VII *b*, was then treated with alkali, and *dl*-oxysparteine was obtained. On dehydrogenation of cytosine with palladium the *norlupinane* nucleus was broken up and afterwards closed, giving, according to Späth (15), 6-methyl-2-hydroxyquinoline. Interesting compounds were obtained by similar treatment of several degradation products of cytosine. α -Norlupinone yielded 8-keto-*ps*-quinolizine-(8), besides conyryne (2-*n*-propylpyridine), the latter being formed by an interesting elimination of carbon monoxide.



¹ Cf. *Ann. Rev. Biochem.*, 4, 499 (1935).

In a similar manner, Tsuda (16) explained the formation (17) of a base, dehydro-decarbonylomatriline ($C_{14}H_{20}N_2$), from matrine. After oxidation of the base, *n*-butyric acid could be isolated, which proved the presence of a *n*-propyl group. Besides α - and β -matrinidine ($C_{12}H_{14}N_2$) the soda-lime distillation of matrine yielded products which after hydrogenation were identified as α -*n*-butylpiperidine and *nor*lupinane. By the method of v. Braun, acetyldihydro- α -matrinidine was treated with cyanogen bromide and after dehydrogenation an intermediate base was obtained, the oxidation of which afforded pyridine-2,3-dicarboxylic acid. Based on these and other experimental data the Japanese authors propose the following formulae for matrine:



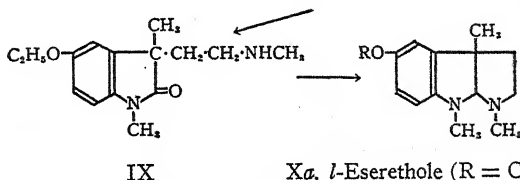
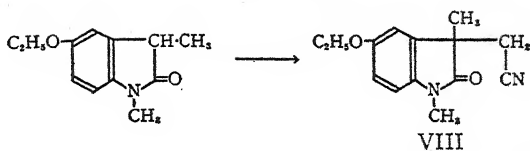
Matrine

Couch (18) isolated a new alkaloid, trilupine, from *Lupinus barbariger*, which he identified with *d*-lupanine-di-N-oxide. This is not the first N-oxide extracted from natural sources as Polonovski showed twenty years ago that geneserine is the N-oxide of its congener, physostigmine (eserine).

Eserine.—Julian & Piki (19) have effected the complete synthesis of physostigmine [from *Physostigma venenosum*; now found in several species of *Dioclea* (20)] in a simple manner, and they believe that the phytosynthesis of this alkaloid is accomplished in a similar way:

The Grignard compound of 5-ethoxy-1,3-dimethyloxindole was permitted to react with chloroacetonitrile. From VIII, after reduction and methylation, 1,3-dimethyl-5-ethoxy-oxindolylethylmethylamine (IX) was obtained, and resolved into its optical antipodes. Further reduction yielded *l*-eserethole (X *a*), which proved to be identical with ethylated eseroline from natural eserine. Synthetic eserethole was de-ethylated by means of aluminum chloride, thus giving *l*-eseroline (X *b*). This phenolic base was converted into physostigmine (X *c*) by Polonovski twenty-five years ago.

A different synthesis of eserethole is recorded by Hoshino (21). The Grignard product of N-methyl-5-ethoxytryptamine (XI) when treated with methyl iodide, furnished *dl*-isonoreserethole (XII) which after methylation yielded *dl*-eserethole. This base has been resolved,

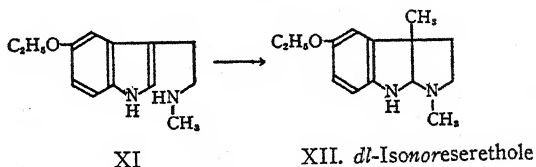


Xa. *l*-Eserethole (R = C₂H₅)

Xb. *l*-Eseroline (R = H)

Xc. Physostigmine (R = CH₃NHCO)

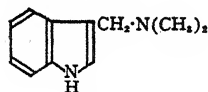
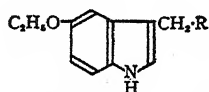
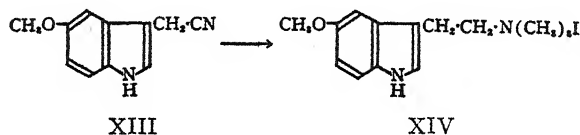
and its methiodide was found to be identical with that of *l*-eserethole. Robinson succeeded in converting *l*-eserethole into *l*-eseroline (22).



Abrine, bufotenine, gramine.—Abrine (C₁₂H₁₄O₂N₂, from *Abrus precatorius*) proved to be an α -amino acid, and, as decarboxylation resulted in N-methyl-tryptamine, abrine itself is N-methyltryptophane (23).

Similar structures have been assigned to some toad poisons, and Wieland supported them by synthesis (24). He submitted 5-methoxy-indole to the Grignard reaction and by means of the Majima method treated the product with chloroacetonitrile. Reduction of XIII, thus obtained, furnished 5-methoxy-indolyethylamine which could be methylated to O-methylbufotenine-methiodide (XIV). This was then rearranged to a phenolbetaine identical with O-methylbufotenidine. A synthesis of free bufotenine is described by Hoshino (25): Ethyl-5-ethoxyindolyl-3-acetate (XV a) was reduced to the corresponding alcohol, the bromide (XV b) of which reacted with dimethylamine to give O-ethylbufotenine (XV c) which could be de-ethylated. Ac-

cording to Euler & Erdtman (26) gramine from certain strains of barley is probably a simple indole derivative, and is identical with the

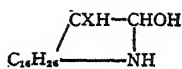


XVa. $\text{R} = \text{COOC}_2\text{H}_5$
 XVb. $\text{R} = \text{CH}_2\text{Br}$
 XVc. $\text{R} = \text{CH}_2\text{N}(\text{CH}_3)_2$

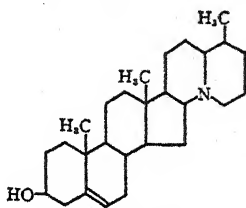
XVI. Gramine

donaxine that Orekhov found (27) in *Arundo donax* (Gramineae). Based on spectroscopic evidence Euler has synthesised several bases of the formula $\text{C}_{11}\text{H}_{14}\text{N}_2$, but none of them was identical with his alkaloid. Wieland, however, succeeded (28) in condensing β -indolyl-MgI with dimethylamino-acetonitrile according to Bruylants' method. The resulting ω -dimethylaminoscatole (XVI) proved to be gramine. Nevertheless, it is not the first alkaloid from the Gramineae as some authors believe. Hordenine was isolated from barley thirty years ago, and it is identical, according to Späth, with anhaline from *Anhalonium fissuratum* (Cactaceae).

Solanidine.—Work on the alkaloids of the Solanaceae has been pursued vigorously. Soltys (29) dehydrogenated solanthrene, a derivative of solanidine-*t* (from potatoes), and obtained methylcyclopentenophenanthrene. This proves that solanidine-*t* is related to the sterol group. The same hydrocarbon has been found on dehydrogenation of solanidine-*s* (from *Solanum sodomaeum*) with selenium [Rochelmeyer (30)]. Both these alkaloids are precipitated by digitonin. From *Solanum pseudocapsicum*, solanocapsine has been isolated and this, according to Barger (31), is related to the alkaloids mentioned above. Little progress has been made in investigations of the basic part of the molecules of these alkaloids. Oddo (32) suggests XVII as the partial formula for solanidine-*s*. A formula of solanidine-*t* mentioned by Soltys may be given as an example of the views held in respect to this group of alkaloids. Clemo expresses a different point of view (33).

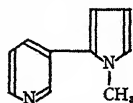


XVII

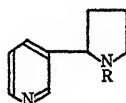
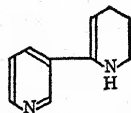
Solanidine-*t* (Soltys)

Nicotine group.—Much exact and important information has been obtained concerning the alkaloids of tobacco. Späth & Kuffner (34a) have hydrogenated nicotyrine (XVIII) to nicotine (XIX *a*), thus simplifying Pictet's synthesis, several stages of which could not be reproduced by Wibaut (34b). In collaboration with Hicks, Späth (35) found *d*-nornicotine (XIXb) in *Duboisia hopwoodii* which is known by the natives of Central Australia as a stimulant. *l*-Nornicotine has been isolated from tobacco in pure form (36), and obtained by the smooth oxidation of *l*-nicotine (37). Furthermore, Späth resolved *dl*-nornicotine into its optically active components, both being natural products (38a).

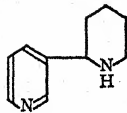
Another base which is a constituent of cigar smoke and important for its aroma is myosmine (m.p. 44°). Though Späth (39) had but little material he succeeded in establishing the constitution of myosmine which has been supported by an assured synthesis (40) similar to the Späth synthesis of nicotine: Ethyl nicotinate was condensed



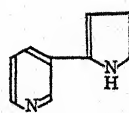
XVIII. Nicotyrine

XIXa. Nicotine (R = CH₃)XIXb. *d*-Nornicotine (R = H)

XX. Anabaseine



Anabasine

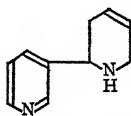


Myosmine

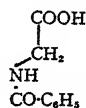
with benzoylpyrrolidone and, on hydrolysis of the condensation product, myosmine was obtained. Späth (40) was able to work out a new

synthesis of *dl*-anabasine: Starting from N-benzoylpiperidone and ethyl nicotinate in the manner given above anabaseine (XX) was obtained, which after hydrogenation yielded anabasine. This racemic base was resolved by means of 6,6'-dinitro-2,2'-diphenic acid (38*b*). The rotation of Nelson's standard *l*-anabasine (41) proved to be erroneous. Späth has discovered (42*a*) some new tobacco alkaloids, trimethylamine, 2,3'-dipyridyl, and piperidine. The latter has been shown by Rimington (43) to be the poisonous principle of *Psilocaulon absimile*. Späth has pointed out that piperidine occurs in the black pepper as a primary product—not produced by the cleavage of piperine (42*b*).

A new alkaloid has been discovered (44) in Kentucky tobacco, termed *l*-anatabine (XXI). It is strongly levorotatory, and is the only tobacco alkaloid the salts of which show the same direction of rotation. On hydrogenation, *l*-anatabine ($C_{10}H_{12}N$) affords *l*-anabasine and 2,3'-dipyridyl. The site of the double bond was determined by oxidation of N-benzoylanatabine, which furnished hippuric acid. An alkaloid that Ehrenstein obtained from the so-called nicotine, and which he believed to be anabasine,² certainly was not anabasine because it differs from it in its optical properties. Ehrenstein's base, like nicotine, seems not to have been uniform, and probably contained *l*-anatabine.



XXI. Anatabine



Hippuric acid

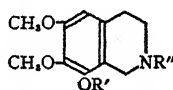
Anhalinine, candicine, pelltine.—Späth isolated two new alkaloids which occur in very small quantity in the mezcal buttons (Cactaceae). One of them, anhalinine, is non-phenolic, and proves to be (45*a*) O-methylanhalamine (XXII*a*), whilst the other, anhalidine, is phenolic. There is synthetic evidence (45*b*) that it is N-methylanhalamine (XXII*b*). An exhaustive collation of the cactus alkaloids, and a new method for their isolation are recorded by Späth (45*c*). A similar list has been given by Reti (46) who mentions two quaternary bases from the Cactaceae not yet known in the European literature, candicine [methylhordeninium hydroxide, XXIII *a*; from *Trichoce-*

² Cf. *Ann. Rev. Biochem.*, 4, 501 (1935).

reus candicans and *lamprochlorus*] and hydroxycandicine (XXIII *b*), the corresponding derivative of dihydroxyphenylethylamine, from *Stetsonia coryne*. According to Reti some other cacti are rich in alkaloids; e.g., *Trichocereus terscheckii* contains 0.3 to 0.4 per cent, which are, however, not physiologically active. By oxidation to isocotarnic acid, and by a synthesis of this acid (47) Späth (45*a*) confirmed the structure of anhalonine which he had already postulated in 1923.

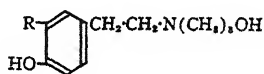
The resolution of pellotine was effected with considerable difficulty (48*a*). As this optically active base is rapidly racemised both in acid and alkaline media, pellotine extracted from natural sources is always inactive. Therefore, it is possible, as Späth believes, that racemisation of pellotine occurs in the living cell. So, contrary to Schöpf, it is not necessary to assume that the biosynthesis of this and similar alkaloids is accomplished without enzymatic interaction.

Späth (48*b*) also resolved synthetically obtained anhalonine and lophophorine.



XXIIa. O-Methylanhalamine
(R' = CH₃; R'' = H)

XXIIb. N-Methylanhalamine
(R' = H; R'' = CH₃)



XXIIIa. Candicine (R = H)

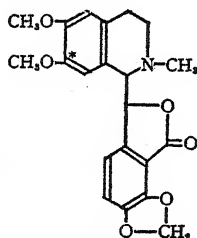
XXIIIb. Hydroxycandicine (R = OH)

Adlumine, *corlumine*, *bicuculline*.—Recent research concerning the alkaloids of *Corydalis* species and related bases resulted in the discovery of about a dozen new alkaloids by Manske (49). One of them, corlumine, yielded on oxidation the same products as adlumine (50). It is, therefore, a stereoisomeride of the latter base (XXIV). Corluminine is one of the two possible monophenolic bases derived from corlumine. Manske prefers the asterisked position for the free hydroxy group. Huang Minlon (51) worked on the alkaloids of the Chinese *Corydalis ambigua*.

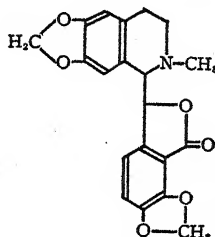
The synthesis of bicuculline, which formerly was elucidated by Manske,³ was accomplished by Robinson (52). Bromonormeconine was converted into 3,4-methylenedioxyphthalide, the 6-nitro derivative of which yielded, on condensation with hydrastinine, a nitro-

³ Cf. *Ann. Rev. Biochem.*, 4, 506 (1935).

bicuculline. The usual methods were then applied for the conversion into bicuculline. The allocation to the stereochemical series, α and β , has not yet been carried out. It may be anticipated that when the



XXIV



Bicuculline

inactive synthetic material has been resolved, the *d*-base will prove to be identical with natural bicuculline.

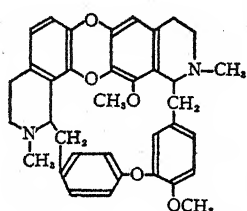
Tetrahydro-isoquinoline group.—Several species of *Papaver* have been investigated by Orekhov (53), and new alkaloids, probably containing the tetrahydro-isoquinoline skeleton, have been discovered. The best known are armepavine, floripavine, floribundine, and floripavidine. Gadamer's statement that isothebaine or thebaine occur in *Papaver orientale*, was not confirmed. Tuduranine (54) seems to belong also to the tetrahydro-isoquinoline type. The alkaloid from *Papaver rhoeas* has been investigated by Späth (55). Awe (56) agrees with his results. A final establishment of the constitution is still lacking.

Formaldehyde condenses with berberine to form 4-methyleneberberine (57) which is an interesting transition from the berberine series to the corydalines. It is possible that this reaction takes place in the living plant.

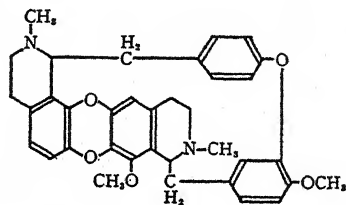
Diphenylether group.—Up to now about eighteen alkaloids are known that doubtless possess diphenyl ether linkages in their molecules. A good summary of these was recently published by Kondo (58). Besides the facts given in earlier reviews isotetrandrine was found to be identical with O-methylberbamine (59*a*), found also in *Berberis vulgaris* (59*b*). According to Kondo (60*a*), *l*-phaeanthine, isolated by Santos from Anonacea a few years ago (60*b*), is the optical antipode of *d*-tetrandrine, whilst cepharanthine is a stereoisomeride of *l*-phaeanthine, the methines being identical (61). Hanfangchine-A, from a Chinese Menispermacea, seems to be identical with tetrandrine

(62a), but contradictory results are also recorded (62b). Finally, Tomita (63) identified O-dimethyltrilobamine with O-methyloxyacanthine.

Three diphenylether linkages coexist in trilobine and isotrilobine, the latter having been considered formerly as homotrilobine (64a).⁴ Faltis (64b) suggests two different structures (e.g., XXV a) for these alkaloids, based on stereochemical considerations. Kondo (58) has discussed different formulae, e.g., XXV b (58). Kondo (65a) has now adopted the formula, O-methylauricine, proposed by Faltis⁵ (65b), and has confirmed the site of the phenolic hydroxy group.



XXVa



XXVb

Menisarine, *normenisarine*, and *insularine* are supposed to contain the diphenyl ether skeleton, and it is possible that a few other alkaloids, e.g., *hanfangchine-B*, belong to the same series.

The formula of *d*-bebeerine (the optical antipode of *l*-curine), suggested by Späth & Kuffner,⁶ has been supported by King (66). King states that the physiologically active principle of the tubocurare, the quaternary base *d*-tubocurarine, yields, on methylation and Hofmann degradation, the same methines as given by O-methylbebeerine. Therefore, he assumes that these bases are stereoisomerides. The position of two methoxy groups has been proved by Späth & Kuffner.⁶ Their suggestion concerning the site of the two other methoxy groups was confirmed by Faltis (67) who succeeded in isolating 4-carboxy-2,2'-dimethoxy-1,1'-diphenyl ether on suitable degradation.

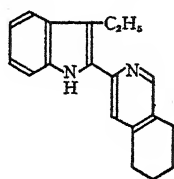
Yohimbine.—According to Wibaut the selenium dehydrogenation of yohimbine affords yobyryne and the so-called tetrahydroyobyryne (XXVI). The latter has been ozonised by Scholz (68) and, after

⁴ Cf. *Ann. Rev. Biochem.*, 2, 440 (1933).

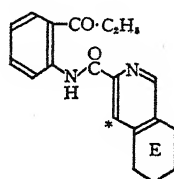
⁵ Cf. *Ann. Rev. Biochem.*, 4, 512 (1935).

⁶ Cf. *Ann. Rev. Biochem.*, 4, 511 (1935).

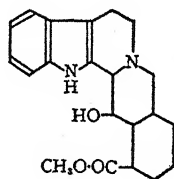
hydrolysis, *o*-aminopropiophenone and an acid ($C_{19}H_{20}O_2N_2$) were obtained. As this acid yields, when oxidised, berberonic acid (pyridine-2,4,5-tricarboxylic acid) it must be 3-carboxy-5,6,7,8-tetrahydroisoquinoline. If this assumption is correct, tetrahydroyobyryne and the product of its ozone degradation (XXVII) must have the structures given below. In connection with the statement that oxidation of yohimbine yields succinic acid, Scholz refuses to assign the OH group to ring E (Hahn)⁷ and prefers the asterisked position. Yohimbine may have the structure, XXVIII.



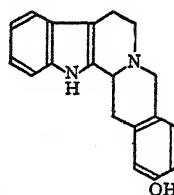
XXVI. Tetrahydroyobyryne



XXVII



XXVIII. Yohimbine (?)



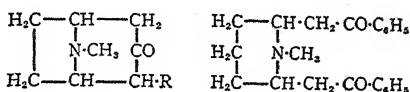
XXIX

Hahn (69) condensed tryptamine with *m*-hydroxy-phenylpyruvic acid at room temperature and at pH 5, and, on decarboxylation, 3-*m*-hydroxybenzyl-3,4,5,6-tetrahydro-4-carboline was obtained; after reaction with formaldehyde, XXIX resulted. According to Hahn this is hexadehydroyohimbol or an isomeride.

Biosynthesis.—The first author who achieved the synthesis of alkaloids at room temperature in dilute and nearly neutral solution was Schöpf. In this way, he obtained new and interesting results. He pointed out (70) that, along the lines of Robinson's classical synthesis, tropinone (XXX a) may be formed in good yield under such conditions, starting from succindialdehyde, methylamine hydrochloride

⁷ Cf. *Ann. Rev. Biochem.*, 4, 505 (1935).

ride, and acetonedicarboxylic acid with spontaneous decarboxylation. In a similar way, monomethyl-acetonedicarboxylate yielded methyl-tropinonecarboxylate (XXX *b*), the latter being important for the biological synthesis of ecgonine. Pseudopelletierine was produced when succindialdehyde was replaced by glutaraldehyde. The same aldehyde was condensed with methylamine hydrochloride and benzoylacetic acid to furnish lobelanine. It is noteworthy, however, that this reaction had to be carried out in acid solution.



XXXa. Tropinone (R = H) Lobelanine

XXXb. (R = COOCH₃)

According to Schöpf, it is out of the question that this manner of synthesis is an exact copy of the natural biosynthesis. Späth, however, is of the opinion that the so-called physiological conditions are but an approximation to those existing in the living cell.

Homopiperonal.—Späth (71) has pointed out that in Hahn's condensation of homopiperonylamine with homopiperonal certainly no derivative of benzyltetrahydroisoquinoline is obtained. It is likely that Hahn's product was a Schiff's base from homopiperonylamine with piperonal, the latter usually being a by-product in the preparation of homopiperonal.

Lycorine, tazettine.—On degradation of lycorine, Kondo (72) obtained methylenedioxy-N-methylphenanthridone which proves that lycorine belongs to the same class of alkaloids as tazettine. Tazettine is, as Späth observed,⁸ a derivative of phenanthridine. Späth (73), in collaboration with Kondo and Orekhov, has identified tazettine with "base VIII" from *Lycoris radiata* and with ungernine from *Ungernia severtzovii*. However, the constitution of lycorine is not yet elucidated.

Veratrine.—Veratridine and cevadine could be found in commercial veratrine. On hydrolysis both gave the same cleavage base, termed cevine, besides veratric and tiglic acids respectively. According to Blount (74) cevine is a tertiary base. It contains one lactone and two hydroxy groups; on distillation with soda-lime it furnishes a

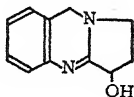
⁸ Cf. *Ann. Rev. Biochem.*, 4, 507 (1935).

volatile base, almost certainly *l*-coniine; on dehydrogenation with selenium a crystalline base is obtained, termed cevanthridine ($C_{23}H_{25}N$), besides a phenolic substance, cevanthrol ($C_{17}H_{16}O$). Both are probably derivatives of phenanthrene. Cevanthridine is supposed to contain a phenanthridine nucleus. From *Veratrum grandiflorum*, Saito & Suginome (75) have isolated jervine but there is little known about its constitution.

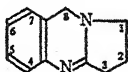
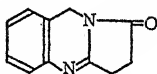
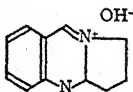
Peganine, vasicine.—After his synthesis⁹ of Δ^9 -pegene (XXXI), Späth (76a) accomplished in a similar way a synthesis of peganine (vasicine), thus establishing the structure of this alkaloid. A little later Adams (77) described a different synthesis of Δ^9 -pegene. Exhaustive reduction of peganine results in a base which Späth has shown to be *o*-aminobenzylpyrrolidine. He found that on smooth acetylation *O*-acetylpeganine is produced whilst on energetic treatment with acetic anhydride *N*-acetylpegadiene is formed (76b). Several ring homologues have been prepared, and new syntheses of Δ^9 -pegene and peganine have been effected (78a) by the condensation of *o*-aminobenzylamine with butyrolactone or hydroxybutyrolactone, respectively. The reaction of amines with aliphatic lactones, which often yields lactames, has been studied using various compounds (79). Furthermore, Δ^9 -pegene was prepared from Δ^9 -pegene-8-one, which is built up by condensation of isatoic anhydride and pyrrolidone (76b). A different synthesis has been reported by Adams (77). Rây & Narang (80a, c) claimed to have synthesised Δ^9 -pegene-1-one (XXXII) and an isomeride of it but Späth pointed out that in both cases the same substance (Δ^9 -pegene-1-one) is formed (81a). Rây & Narang stated that by electrolytic reduction of their isomeric products pegane and an isomeride of pegane are produced. Späth (81b) obtained experimental evidence that under these conditions neither pegane nor an isomeride is formed but *o*-aminobenzylpyrrolidine, the properties of which are in good agreement with those of the products obtained by Rây & Narang. The Indian authors (80c), finally, have accepted the views of Späth. Späth has also described the isolation of optically active peganine from *Adhatoda vasica* (82), and has resolved the racemic form of this alkaloid (2). Rosenfeld & Kolesnikov claim to have published the isolation of *l*-peganine from *Peganum harmala* in a paper not accessible in Europe (cf. 83). Schöpf has accomplished the interaction of *o*-aminobenzaldehyde with γ -amino-

⁹ Cf. *Ann. Rev. Biochem.*, 4, 502 (1935).

butyraldehyde under his "physiological conditions" and succeeded in rearranging the primary product (XXXIII) to Δ^9 -pегene (84).

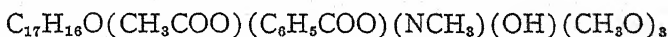


Peganine (vasicine)

XXXI. Δ^9 -PегeneXXXII. Δ^9 -Pегene-1-one

XXXIII

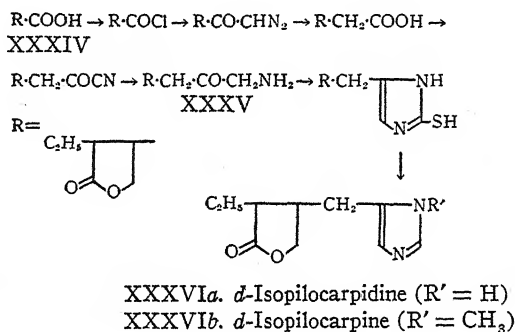
Aconitine.—Freudenberg (85) proved that destructive distillation over barium hydroxide of the by-products from the manufacture of pure aconitine yields *l*-ephedrine. Aconitine, itself, on fusion with alkali is broken down in a different way; ethylamine is evolved, but *l*-ephedrine cannot be detected (86); the Herzig-Meyer method yielded ethyl iodide, identified as ethyltrimethylammoniumiodide. A new oxidation product of aconitine, termed aconitoline, is described by Lawson (87). Its formula is supposed to be



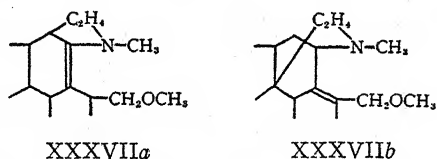
Majima (88) treated derivatives of aconitine, its lower homologue (mesaconitine), and an oxidation product of both (oxonitine) with amylalcoholic potassium hydroxide and obtained ethylamine, methylamine, and ammonia, respectively. On oxidation, aconitine gave acetaldehyde, whilst mesaconitine yielded formaldehyde. It seems that oxonitine contains no N-alkyl group. If one adopts the formula $\text{C}_{34}\text{H}_{47}\text{O}_{11}\text{N}$ for aconitine, oxonitine may be considered as $\text{C}_{32}\text{H}_{41}\text{O}_9\text{N}$; this formula was first discussed by Späth (89). Freudenberg (86b) pointed out that there are six rings combined in the aconine molecule.

Pilopic acid, pilocarpine.—Tschitschibabin (90) succeeded in synthesising pilopic and isopilopic acids, which are the stereoisomeric ethylparaconic acids. The acids were separated and resolved (XXXIV). Preobrashenski (91) converted isopilopic acid into homoisopilopic acid along the lines of Arndt's (92) method. The chloride of *d*-homoisopilopic acid was treated with diazomethane, and, after several intermediate reactions, a base (XXXV) was obtained, which

on treatment with potassium thiocyanate furnished mercapto-*d*-homo-isopilopyl-iminazole. Oxidation with potassium ferricyanide resulted in *d*-isopilocarpidine (XXXVI *a*) which, by Späth's method, was methylated into *d*-isopilocarpine (XXXVI *b*). The synthesis of *d*-pilocarpine proved to be more difficult because all members of the pilopic series easily rearrange to the corresponding iso compounds.

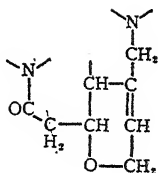


Strychnine.—There is steady progress to report in the elucidation of the *Strychnos* alkaloids. The opinions of Leuchs and Robinson differ with regard to the site of the double bond in the neo series. Whilst Leuchs prefers the formula XXXVII *a*, Robinson (93) adheres to his previous formulation, XXXVII *b*. The position of a C_2H_4 group is still under discussion. On fusion of strychnine or certain

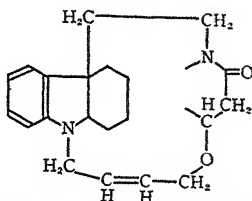


derivatives with potassium hydroxide, Kotake (94) obtained tryptamine. This, and further new information, enabled him to suggest that the groupings XXXVIII or XXXIX occur in this alkaloid—in contradiction with the formulae of Robinson and Leuchs. These authors have not yet commented on Kotake's findings. Clemo (95) degraded strychnine with alcoholic potassium hydroxide, and obtained several simple bases, one of which seems to be the indolenine form of

tryptamine; ordinary tryptamine could be changed reversibly into this base.



XXXVIII

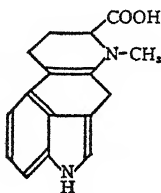


XXXIX

Ergot group.—The ergot alkaloids are known in two isomeric forms which can be converted into each other. Whilst one of them possesses strong physiological activity, and is levorotatory, the other is dextrorotatory and almost inert. On hydrolytic fission, the two related alkaloids, ergotinine and ergotoxine, yield lysergic acid and ammonia, together with isobutyrylformic acid, proline, and phenylalanine (96a). On summation of these components, with elimination of water, the correct empirical formulae for the alkaloids are obtained. On similar treatment, ergotamine and its isomeride ergotaminine give the same products except that isobutyrylformic acid seems to be replaced by pyruvic acid. This would agree with the analytical figures (96b, c).

Perhaps the most important result in the field of the ergot alkaloids is the isolation of ergometrine by Dudley & Moir (97). This new base is soluble in water, and shows a rapid and strong, but rather fleeting activity. It is slightly toxic. Stoll (98) described and analysed the base [which was termed also ergobasine, ergotocine, and ergotetrine (99)] in a pure state. Ergometrine, and its isomeride ergometrinine (100), have the composition $C_{19}H_{18}O_3N_2$, and prove to be the hydroxy-isopropylamide of lysergic acid (101). As the two corresponding isomerides in each case yielded the same cleavage products the question arose as to the difference between them. The answer was disclosed by the work of Jacobs & Craig, and Smith & Timmis (102). Different dihydrolysergic acids were obtained on hydrolysing the hydrogenated levobases or their isomerides. Therefore, the lysergic acid component of the alkaloids must be different though under the conditions of hydrolysis only one form proves to be stable. Lysergic acid could be isomerised to isolysergic acid.

The problem of fundamental importance in the investigation of these ergot bases is the elucidation of the structure of lysergic acid. Several methods of degradation have been applied but only a few cleavage products are exactly identified. The most important are quinoline, 2-methyl-3-ethylindole, and 1-amino-5-methylnaphthalene. The formula given (XL) is suggested by Jacobs & Craig (102) but



XL

it is far from certain. A further pair of isomerides has been detected, termed ergosine and ergosinine (103). Finally, a new alkaloid, ergomonamine ($C_{19}H_{19}O_4N$), has been isolated, which differs in its composition and properties from the lysergic acid derivatives (104).

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PHOTOSYNTHESIS*

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THE INCONSTANCY OF PHOTOSYNTHESIS UNDER NATURAL CONDITIONS

Photosynthesis has often been found to follow a capricious and inconstant course quite unrelated to external conditions supposed to govern its rate [cf. Harder (49) ; Jaccard & Jaag (53) ; Ehrke (22) ; Kurssanow (65) ; Drautz (21)]. The opinion is not uncommon that photosynthesis in the higher plants, especially under "natural conditions," is governed only indirectly by the usual external factors, through a complicated series of stimulatory reactions. From his experiments with algae van der Paauw is inclined to regard the influence of external factors as largely indirect (76, p. 502-509; also 78). At the same time there are a number of outstanding investigations indicating that photosynthesis is governed directly by certain external factors, notably carbon dioxide tension, light intensity, and temperature. Among earlier work may be mentioned that of Willstätter and Stoll on the leaves of higher plants, and that of Warburg on *Chlorella*. In both cases, the modifying influence of internal factors was evident, and their importance is not denied. Among more recent experiments are those of Mitchell (69) with geranium, primula, wax beans, tomato, and cabbage. He reports variations in rate of photosynthesis no greater than 14 per cent over periods of seven hours. The rate was remarkably independent of stomatal aperture and relative humidity, and there is every indication of direct control of photosynthesis by external factors, which were carefully regulated.

It is more than likely that photosynthesis is controlled by the same external factors in the majority of green plants. The leaf of a higher plant, with its vascular connections, is a far more complex system than a unicellular alga, and it is not surprising that careful technique is necessary to demonstrate the influence of external factors on one process, photosynthesis, when these same factors are known to influence other processes in the leaf which in their turn

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may affect the rate of assimilation (opening and closing of stomata, transport of materials, etc.). If it can be shown by satisfactory experiments that assimilation is sometimes unrelated to these external factors, it would be well to look first for internal processes which may merely obscure the direct effect of the external factors, rather than to doubt the existence of direct effects. For instance, in the experiments of Jaccard & Jaag (53) photosynthesis was measured by carbon dioxide consumption. This was observed to fluctuate widely. Evidently at least some of the leaves they used were capable of storing carbon dioxide independently of photosynthesis, for they absorbed considerable amounts in the dark. This being the case, carbon dioxide absorption was probably not a true measure of photosynthesis in the light, which may have maintained a constant rate in spite of fluctuations in absorption.

Inspection of the experiments with leaves of higher plants gives one the impression that those investigators who have worked with detached leaves have generally been more successful in obtaining constant rates of assimilation under constant external conditions. The unaccountably fluctuating rates have usually come from experiments with entire plants, or with leaves attached to entire plants. In these experiments inferior methods have often been used, but we know too little about the physiological effects of separating a leaf from its parent plant to judge with any certainty whether the difference is real or results from the use of superior methods by those who have been willing to sacrifice natural conditions to the extent of working with detached leaves. It should be noted, however, that Mitchell's remarkably uniform results were obtained with leaves attached to entire plants.

In simpler organisms external conditions come closer to the plastid, the real seat of photosynthesis, and from them comes most of our concise knowledge of the process, because in these cases it is evidently a direct function of external conditions. Recognizing that man's interest must center more particularly on those organisms most important in the economy of the world about him, and on the conditions under which they naturally grow, it is nevertheless essential to keep in mind the results of studies on organisms whose carbon dioxide assimilation is directly dependent on external factors. Though they may be economically insignificant, they are likely to be helpful in unravelling some of the baffling complexities of assimilation encountered in the higher plants, provided we do not make separate problems of

photosynthesis under natural and artificial conditions. The solution of the broader problems involved under natural conditions will depend on a fundamental understanding of the simpler and more artificial conditions. Believing that there is current considerable misunderstanding concerning what is at the present time our most fundamental knowledge of photosynthesis, the reviewer has tried to clarify some of the more important issues. While many of the details presented may appear superfluous to the casual reader, the issues involved would escape satisfactory interpretation without them.

CHEMICAL AND KINETIC MECHANISMS

Recently various attempts have been made to integrate the scattered and incomplete physiological data available concerning chlorophyll photosynthesis, by constructing either chemical or kinetic mechanisms. Several of the proposed chemical mechanisms [Willstätter (91); Stoll (88, 89); Franck (29, 30); James (54); Shibata & Yakushiji (85)] have fundamental features in common and may be discussed together. It is supposed that carbon dioxide must combine with chlorophyll in order to be reduced. Reduction to formaldehyde then takes place in several steps, requiring the successive absorption of four quanta of visible light. As a result of the rearrangements required to obtain formaldehyde from carbon dioxide or carbonic acid, some sort of peroxide is formed. Its decomposition with the liberation of oxygen by the enzyme catalase is supposed to constitute the Blackman reaction. Franck (29) has put forward what appears to be the most rational sequence of photochemical steps, and made an especial effort to interpret the changes in fluorescence intensity observed by Kautsky (57 to 62) in experiments with living leaves. Kautsky and his collaborators report that oxygen, alone of all the gases tried, acts as a powerful quenching agent for chlorophyll fluorescence. To explain this, Franck has proposed that chlorophyll must be partially photo-oxidized before the energy it absorbs can be used for carbon dioxide reduction.

Mechanisms of this type have been discussed in detail by Emerson (24, part III). It is coming to be generally recognized that the large amount of chlorophyll present in the average leaf or plant cell in proportion to the amount of carbon dioxide reduced per unit time makes it unlikely that the assimilatory process requires the combina-

tion of chlorophyll with carbon dioxide. Various earlier experiments can now be shown to point to the same conclusion, but attention was drawn to this relationship by Emerson & Arnold's (25) determination of the ratio of chlorophyll content to amount of carbon dioxide reducible by an instantaneous light flash of saturating intensity. The flashes were spaced far enough apart so the Blackman reaction was not a limiting factor. According to their measurements, *Chlorella* cells contain about 2500 chlorophyll molecules for each reducible carbon dioxide molecule. It can be shown from other data [cf. Gaffron & Wohl (44, p. 86)] that at light intensities giving moderate rates of photosynthesis it may be a matter of minutes before each chlorophyll molecule has absorbed a single quantum. If a single chlorophyll molecule must absorb four quanta to complete the reduction of one molecule of carbon dioxide, Kohn (64) calculates that it would be a year before a given cell suspension could do a measureable amount of photosynthesis. Yet experiment shows that such a cell suspension gives easily measurable photosynthesis in five-minute periods. If chlorophyll is combined with carbon dioxide, then quanta absorbed by more than one chlorophyll molecule must certainly be available for the reduction of a single carbon dioxide molecule. It is more probable that no such compound plays any direct part in photosynthesis.

The general belief that the Blackman reaction is a peroxide decomposition is founded on the similarity between the Blackman reaction and the decomposition of hydrogen peroxide carried on in the dark by *Chlorella* [Warburg & Uyesugi (90); Yabusoe (92); Yakushiji (93)]. Hydrogen peroxide decomposition in *Chlorella* has been reinvestigated by French (32) and by Emerson & Green (27). In both cases the results differed in important details from those originally obtained in Warburg's laboratory. Emerson & Green, making a closer comparison with the Blackman reaction, conclude that while their results cannot definitely refute the theory that the Blackman reaction is a peroxide decomposition, they show the experiments of Warburg and his collaborators should not be interpreted as supporting it.

Kautsky's observations and conclusions regarding the rôle of oxygen in photosynthesis have been severely criticized by Emerson (24, p. 336) and by Gaffron (36, 38, 41, 42, 43), on both experimental and theoretical grounds. There are reasons for doubting that the changes observed by Kautsky have anything to do with photosynthesis. Franck & Wood (31), while disagreeing with Kautsky's in-

terpretation, believe oxygen may nevertheless play a more or less direct part in the process. They have confirmed Kautsky's qualitative observations, and extended them with quantitative measurements of chlorophyll fluorescence in leaves and in organic solutions. Recognizing now that Franck's earlier hypothesis of partial photo-oxidation of chlorophyll as a preliminary step in assimilation is untenable, they suggest the observed changes in fluorescence are due to photo-oxidation of organic substances adsorbed on the chlorophyll. In the leaf these substances, which may be intermediate and final products of photosynthesis or other metabolic products, compete with carbon dioxide for the chlorophyll surfaces, and inhibit photosynthesis. Their removal by photo-oxidation promotes assimilatory activity. Light saturation, Franck suggests, may be a matter of balance between assimilation and photo-oxidation. In support of this interpretation he cites Gaffron's (41) observation that lack of oxygen in the dark is followed by diminished oxygen production in photosynthesis. However, under all ordinary conditions Gaffron found prolonged exposure to complete lack of oxygen was quite without effect on subsequent photosynthesis. Only in unusually dense suspensions was he able to demonstrate diminished oxygen production. To the reviewer this experiment of Gaffron seems inconclusive for several reasons, but especially because no precautions appear to have been taken against the development of anaërobic bacteria during the periods of anaërobiosis which lasted for many hours. In this laboratory we have failed to find any inhibitory effect following exposure of moderately dense cell suspensions to lack of oxygen in the dark under sterile conditions. Possibly the toxic substances which Gaffron believes were responsible for his results were produced by bacteria rather than by assimilating cells.

Objection to Franck's newest interpretation of fluorescence changes must also be made on the ground that it calls for long induction periods at low light intensities, contrary to the known behavior of photosynthesis. His interpretation of light saturation has but little physiological basis. Nevertheless, the observations of Franck & Wood suggest that valuable information regarding the functions of chlorophyll in photosynthesis may be gained from a study of photo-oxidations carried out by chlorophyll *in vitro* and the changes in fluorescence which accompany these processes as well as assimilation.

Looking at the subject from a different angle, Spoehr & Smith (87, p. 1049) see in the long lifetime of activated oxygen a possibility that it may play a direct part in the transference of energy.

Van Niel (73, 74) has suggested that since four hydrogen atoms would suffice to reduce one molecule of carbon dioxide to formaldehyde, and since four light quanta are apparently the minimum number which must be absorbed in order to effect this reduction, the primary action of the light may be to produce active hydrogen, one atom being activated for each absorbed quantum. He regards photosynthesis as essentially a case of hydrogen transference because the stoichiometric equations for the process in the purple bacteria are most easily interpreted in this way. These organisms reduce carbon dioxide but produce no oxygen, despite the fact that they are abundantly supplied with catalase [Gaffron (39, p. 304)]. This is of interest in connection with the supposed function of catalase in chlorophyll photosynthesis, discussed above. The purple bacteria require special hydrogen donors such as oxidizable sulfur compounds, fatty acids, or even gaseous hydrogen [Gaffron (35, 37, 40); Roelofsen (81, 82); van Niel (73, 75)], while the green plants use water as a hydrogen donor. Photosynthesis in both cases is regarded as a hydrogenation of carbon dioxide, rather than a reduction of carbonic acid.

In the results of recent measurements of quantum yield in the purple bacteria (discussed below) there is some support for van Niel's approach. These organisms may prove to be limited to the same quantum yield as the green plants, although it should be thermodynamically possible for them to reduce carbon dioxide with considerably less energy, because less is required to obtain hydrogen from the donors used by the purple bacteria than from water, to which the green plants seem to be restricted.

The mechanism proposed by van Niel is an important advance over others because it does not require a complex sequence of photochemical reactions such as are called for if four quanta must be absorbed by a single chlorophyll molecule in order to reduce one molecule of carbon dioxide. Instead, each absorbed quantum is supposed to do the same thing—produce one active hydrogen. But it must be remembered that we have as yet no indication whether the cell assimilates by hydrogenating carbon dioxide or reducing carbonic acid, and there are some objections to assuming that the absorption of light by green plants produces active hydrogen atoms.

Gaffron & Wohl (44, p. 88, 103), recognizing that carbon dioxide probably does not combine with chlorophyll preparatory to assimilation, suggest it may combine with a free amino or imino group, though

they have no evidence for this. They discuss in some detail the ratio between chlorophyll content and reducible carbon dioxide established by Emerson & Arnold (25) for *Chlorella*, and seek to show that it represents a real entity in the photosynthetic mechanism. They have taken data from the work of various investigators and compared the estimated number of quanta absorbed per second per chlorophyll molecule with the rate of carbon dioxide reduction. In most cases neither the intensity in terms of quanta nor the degree of absorption was accurately known. According to their estimates, the time required for one chlorophyll molecule to absorb four quanta would be too long to accord with the mean time for reduction of a molecule of carbon dioxide calculated by Emerson & Arnold (cf. 44, p. 89, Table I). They conclude that about a thousand chlorophyll molecules must work together to effect the reduction of one carbon dioxide molecule. In consideration of the approximations made in calculating the rate of absorption of quanta [in one case certainly incorrect, cf. Gaffron & Wohl (45)], their value may be said to agree well with Emerson & Arnold's figure of 2500. They represent it as an independent confirmation of the reality of the chlorophyll unit, but their method of arriving at it appears to depend directly on Emerson & Arnold's determination.

Whether groups of chlorophyll molecules act together in photosynthesis is an important question to which the calculations of Gaffron & Wohl offer no convincing answer. Arnold & Kohn (7) share their opinion that a real unit exists, and have measured its size in a number of different organisms. They state in the summary of their paper that it lies between two and three thousand in all cases, but values up to four and five thousand are shown in Table III of their paper. Their purpose was to establish the order of magnitude of the unit, rather than its precise value. It would help to decide the nature of the unit if we knew whether it varied at random, and within what limits. This will require more careful application of the technique than was made by Arnold & Kohn.

Emerson (24, p. 322) thinks it more probable that the so-called chlorophyll unit represents the ratio between chlorophyll and some other internal factor, perhaps the substance which combines with carbon dioxide. The measurements themselves show only that the number of chlorophyll molecules greatly exceeds the number of instantaneously reducible carbon dioxide molecules. Since it has long been believed that carbon dioxide combines with chlorophyll in photosyn-

thesis, we have sought to relate these two numbers. It would be more reasonable to dismiss the chlorophyll from discussion, and look for something in the cell in amounts commensurate with the reducible carbon dioxide, because, as has been explained above, there are now good reasons for doubting that carbon dioxide combines with chlorophyll, at least as a step in assimilation.

Kinetic mechanisms.—Ultraviolet radiation (2537 Å) damages the photosynthetic mechanism of *Chlorella* irreversibly without influencing respiration [Arnold (4)]. From a comparison of this inhibition in continuous and flashing light Arnold (5) predicts that any successful mechanism for photosynthesis will have to express the Blackman reaction as a first-order process. He has proposed (6) a kinetic mechanism for *Chlorella* photosynthesis which reproduces most of the experimental curves in both continuous and flashing light. Other mechanisms have been devised by Briggs (13), Baly (8, 9, 10), Burk & Lineweaver (14, 15), and Smith (86). The mechanism of Burk & Lineweaver is perhaps the most general. It can be adjusted to reproduce accurately the curves of many different physiological experiments.

Some of the objections to the use of kinetic mechanisms in the study of physiological processes have been mentioned by Boysen-Jensen in a discussion of the mechanism proposed by Burk & Lineweaver (cf. 14, p. 180). Prediction of the course of a biological process such as photosynthesis, where nearly all the reactants are still unknown, is relatively easy because of the absence of restrictions on the choice of assumptions, and it is small wonder that the experimental curves can be fitted pretty accurately by a variety of equations. James (55) has pointed out the present inaccessibility of most of these assumptions to experimental verification. Owing to their purely speculative character, kinetic mechanisms have so far failed to exert a directive influence on the course of research in photosynthesis, or in the development of our understanding of the process. Prediction of the course of a biological process by means of equations may not always be helpful in elucidating the reactions actually involved.

THE QUANTUM EFFICIENCY OF PHOTOSYNTHESIS

In a paper dealing with the influence of cyanide on the photosynthetic process, van der Paauw (79) has raised the same objection

brought forward earlier by Briggs (12) against the efficiency measurements of Warburg & Negelein with *Chlorella*. He suggests that less energy may be stored in photosynthesis below the compensation point than above, because below, where the measurements of Warburg & Negelein were made, carbohydrates may be formed by the reduction of intermediate products of respiration, instead of from carbon dioxide and water. Spoehr & Smith (87, p. 1048) have set aside Briggs' objection as being irrelevant to the real purpose of the efficiency measurements, but it is nevertheless of great importance to establish whether carbon dioxide or some less oxidized compound is reduced during photosynthesis below the compensation point, and whether the same quantum efficiency is obtainable above as below the compensation point.

Since the experiments of Warburg & Negelein fifteen years ago, there has been no further work of unquestionable quality on the quantum efficiency of green plants. The assumption that chlorophyll photosynthesis in general must be capable of the maximum quantum efficiency found for *Chlorella*, has played a decisive part in papers on chemical and kinetic mechanisms. Yet the high quantum yields obtained by Warburg & Negelein were not even a general characteristic of their *Chlorella* samples. A particular sequence of lighting conditions proved to produce cultures of cells giving high yields. Whether the characteristics developed in *Chlorella* by these culture conditions prevail generally in green plants is not known. In experiments with foliage leaves or with other organisms more difficult to work with than *Chlorella*, poor physical technique has made it impossible to determine whether the low efficiencies obtained were characteristic of the organism, or resulted from the difficulties involved. Probably the efficiency of the photosynthetic apparatus in any organism depends on previous culture conditions. The apparent impossibility of duplicating Warburg & Negelein's efficiency figures with more complex material should not be attributed entirely to the experimental difficulties. In order to make progress in this work, an organism must be chosen which is suited to the available physical technique, and the experimental difficulties must be adequately met. The researches of Burns (16) and of Gabrielsen (34), while they make interesting contributions to our knowledge of the qualitative behavior of higher plants in different colors of light, fail to touch the problem of quantum efficiency because of the inadequacy of their technique in combination with the organisms with which they have chosen to work.

Roelofsen (82, chapter 5), in an extensive study of photosynthesis in the purple sulfur bacteria, has measured quantum efficiency in several wavelengths of the visible spectrum extending from the sodium line to the blue line of mercury. He concluded that the light absorbed by the red pigment was not available for assimilation. The highest efficiency obtained was one molecule of carbon dioxide reduced for about seven absorbed quanta. Allowing for some loss due to absorption by the photochemically inactive red pigment, he considers it not unlikely that the minimum requirement is four absorbed quanta per molecule of carbon dioxide, in spite of the fact that one quantum of near-infrared light should be more than sufficient thermodynamically for the chemical change involved. He sees in his results support for van Niel's belief that the photochemical process in purple bacteria is the same as in green plants.

French (private communication) used a representative of the *Athiorhodaceae* capable of reducing carbon dioxide with gaseous hydrogen as hydrogen donor, and measured quantum yields in infrared light, where absorption by the red pigment is minimal. His highest efficiencies approach the value of four absorbed quanta per molecule of carbon dioxide, and like Roelofsen he concludes there is a close relationship with the corresponding process in green plants.

Although the physical technique used by French and Roelofsen appears to have been good, there are some unsatisfactory points in both cases. The nature of the dark reaction was unknown, and may not have been the stoichiometric reverse of photosynthesis, as respiration is of chlorophyll photosynthesis. Assimilation was measured manometrically, and a given pressure change below the compensation point may have an entirely different meaning from above. In a discussion of the complex chemical changes carried out by the purple bacteria, van Niel (75, part 3) has shown what serious errors may be introduced by using the manometric method, which is only suitable for metabolic experiments in which the gas exchange has been definitely established by analytical methods. French's treatment of his manometric results is particularly disturbing because the intensity curve is sigmoid, instead of reaching its maximum slope as it approaches the ordinate axis. French has calculated efficiencies from the maximum slope of his curves, out near the region of inflection, and this gives a higher figure than the quantum yield actually prevailing. By pretreating the cells in certain ways he was able to make the maximum slope come nearer to intersecting the origin, and a limiting

value of four quanta per molecule of carbon dioxide reduced appears to have been approached. French's method of handling the data may be the best possible under the circumstances, but the quantum yield obtained in this way is not directly comparable with Warburg & Negelein's figure for *Chlorella*, which was obtained in a more direct way.

French's use of very high partial pressures of hydrogen during his efficiency measurements was ill-advised, as shown by some of the experiments with different kinds of pretreatment. The hydrogen seems to have been somewhat injurious in high concentrations.

The maximum possible efficiency obtainable from the purple bacteria may not yet have been reached, and it remains an open question whether the photochemical process has the same mechanism as in green plants; but it is interesting that photosynthesis has not yet been achieved with less than four absorbed quanta per molecule of carbon dioxide.

THE QUESTION OF PHOTOCHEMICAL ACTION ON THE PART OF THE CAROTENOIDS

Montfort (71) has re-examined the evidence for the common belief that the carotenoids play no photochemical part in assimilation, and rejected it as insufficient. Having discussed Warburg & Negelein's conclusion that the yellow pigments show activity, he reports experiments of his own which he says prove conclusively that light absorbed by these pigments is used for photosynthesis. The importance of this question is such that Montfort's paper is considered in some detail.

The removal of the blue and green portions of the spectrum sometimes results in a decrease in the rate of photosynthesis, and this has been interpreted as indicating that the carotenoids, whose visible absorption is appreciable only in the blue and green, are photochemically active. Montfort (71, p. 731) explains that this is probably due to the use of subsaturating light intensities, because there is no doubt that normal maximal photosynthesis is possible in light containing no wavelengths absorbed by the yellow pigments. This established fact is the basis of various broad statements that the carotenoids are photochemically inactive, at least as far as carbon dioxide assimilation is concerned, but Montfort evidently realizes that it proves only that if they are photochemically active, their activity is not essential.

For positive evidence of their photochemical activity Montfort

turns to the quantum yields reported by Warburg & Negelein for blue light. When computed according to the Einstein photochemical equivalent law, these were a little low in comparison to the yields in wavelengths not absorbed by the yellow pigments. On the other hand, if the estimated portion of the blue light absorbed by the yellow pigments was deducted from the input energy, and the yield computed on the basis of the energy absorbed by chlorophyll alone, it became too high in the blue, in comparison with the longer wavelengths. They concluded that the light absorbed by the yellow pigments must be available for photosynthesis, but at lower quantum efficiency than the light absorbed by chlorophyll.

There are other possible explanations which are not discussed either by Warburg & Negelein or by Montfort. The proportion of blue light absorbed by the carotenoids was measured in a homogeneous solution of extracted pigments, and it may be that this figure should not be applied to the pigments in the plastid, because their distribution may be such as to favor absorption by chlorophyll. It is also conceivable that the quantum efficiency of the chlorophyll is higher in the blue than in the longer wavelengths. In either case it would be unnecessary to assume photochemical activity on the part of the yellow pigments in order to interpret Warburg & Negelein's results. Montfort, however, favors their original interpretation, and seeks to confirm it with similar measurements on organisms having a much larger proportion of carotenoids, the brown marine algae. His technique for determining the rate of photosynthesis has been criticized by Emerson & Green (26). It certainly does not have the refinement we should expect for measurements of quantum efficiency, even though only relative values were desired. His use of broad spectral regions instead of monochromatic light would make the comparison of the absorbed energy in the various colors difficult even for the best experiments.

But these matters are of minor importance compared with the error Montfort has introduced in estimating the relative amount of energy absorbed in the different colors. He measured photosynthesis in different colors of light of equal energy content. Then he assumed the absorbed energy in each color was proportional to the relative absorptive capacity of the alga for that particular color. This is a satisfactory approximation when the absorbed energy is small in comparison to the incident energy, or when absorption and assimilation are measured on samples of equal density. There is no assurance

that either of these provisions was fulfilled, except in experiments with the relatively transparent *Ulva* thallus, where Montfort found relative efficiencies in red and in green light which are in fair agreement with Warburg & Negelein's absolute values. In the case of the more opaque thallomes of the brown algae, he found relatively high efficiencies in the colors absorbed by the carotenoids. Probably this is because his assumption leads to estimated values of absorbed energy which are relatively quite incorrect. To take an extreme case, let us suppose that a piece of material, so opaque that it absorbs all the incident light, is used for photosynthesis measurements in red and green light of equal intensities. The same amount of energy will be absorbed in each color, but if the relative absorptive capacity was determined for a more transparent sample (possibly necessary in order to obtain sufficient transmitted intensity for the measurements), and was found to be ten times as great in red as in green, Montfort's assumption would lead us to suppose that the energy absorbed by our opaque sample was only one-tenth as much in green light as in red, and this would lead to a relatively high efficiency in green light. Since many of the denser marine algae must absorb as much as 90 per cent of the incident light in some of the colors in question, and much less in others, Montfort's efficiency figures may not be even relatively correct, and cannot serve as evidence for photochemical action on the part of the carotenoids.

Montfort is right in thinking that relative efficiency measurements would help to establish whether the accessory pigments are photochemically active. The reason we do not have an abundance of such measurements is that the estimation of the relative amount of energy absorbed in different wavelengths is impossible in most cases.

Extensive work on photosynthesis in marine algae has been done by Seybold (83), as well as by Montfort (70), primarily to determine the influence of wavelength and intensity on the distribution of various forms, and the activity of accessory pigments. The complexity of the factors involved calls for better experimental technique than has so far been applied to these problems. The difficulty of maintaining an adequate concentration of carbon dioxide in a small sample of sea water without sacrifice of physiological conditions has been discussed by Emerson & Green (26), who were only partially successful in solving the problem. Seybold and Montfort do not seem to have taken this factor, as well as some others, sufficiently into consideration, and the results are certainly not decisive.

THE CARBON DIOXIDE FACTOR AND SOLARIZATION

Van den Honert (52, p. 231) has objected to the use of carbonate-bicarbonate mixtures as suspending fluid to provide carbon dioxide for photosynthesis experiments. These mixtures are useful because they maintain constant partial pressures of carbon dioxide at almost any desired concentration for experiments of moderate duration. Their disadvantages are that the pH varies widely for the different carbon dioxide concentrations, that the constants required for the calculation of these concentrations are not accurately known, and that the mixtures are injurious to some kinds of cells. Water plants are supposed to take up either dissolved carbon dioxide or carbonic acid, and the concentrations of carbonate and bicarbonate are supposed to play no part in determining the rate of assimilation, but recent experiments of Arens (1, 2, 3) make this doctrine doubtful. He has shown that leaves of water plants may absorb both carbonate and bicarbonate through their lower surfaces and excrete carbonate or hydroxyl ions through their upper surfaces. The polar transport of the metal ions and the preferential absorption of combined carbon dioxide by the lower surfaces have been demonstrated by using the leaf as a septum between two different solutions. Transport takes place only in the light, and is dependent on assimilatory activity.

If it can be shown that organisms like *Chlorella* can use combined as well as free carbon dioxide for photosynthesis, the usual interpretation of experiments in carbonate mixtures will be subject to considerable revision.

The influence of pH has been investigated by Emerson & Green [cf. Emerson (24, p. 313)], but only at carbon dioxide saturation. Here the rate of photosynthesis remained constant from pH 5 to 8. Unfortunately they were unable to extend their experiments to more alkaline values and to limiting carbon dioxide concentrations because of inapplicability of their methods. Their results show only that the maximum rate of photosynthesis is independent of pH and bicarbonate concentration from pH 5 to 8.

Both van den Honert (52) and van der Paauw (76) found that *Hormidium* photosynthesis differed in certain respects from *Chlorella* photosynthesis as described by Warburg. Most of the experiments with *Chlorella* have been done in carbonate mixtures, while culture media have been used for *Hormidium*. In comparing the two methods

as applied to *Hormidium*, van der Paauw (77) found that the carbonate mixture was definitely injurious to photosynthesis. This is not the case with *Chlorella*. He concluded that the differences in results were due to physiological differences between the two organisms rather than to the methods. He considered the latter to be of equal value, except that manometric experiments in carbonate mixtures were more rapid and convenient. He overlooked the fact that the manometric method of Warburg is equally applicable to experiments in culture fluid. Here, however, it is restricted to saturating concentrations of carbon dioxide, just as is his own method. To the reviewer it seems that the more important differences between *Chlorella* and *Hormidium* are those appearing at limiting concentrations of carbon dioxide. Here we have only van den Honert's experiments in culture medium to compare with Warburg's in carbonate mixtures. While there are undoubtedly physiological differences between *Hormidium* and *Chlorella*, it seems likely that the factors controlling photosynthesis operate in much the same way in both cases. When proper methods are developed, it will probably turn out that van den Honert's experiments in culture medium give a more correct picture of the influence of the carbon dioxide factor than do Warburg's in carbonate mixture. However, the interpretation of experiments in carbonate mixtures giving subsaturating concentrations of carbon dioxide is at present uncertain, in view of the work of Arens mentioned above.

Burr (18) suggested that carbonic anhydrase, an important enzyme found in red blood corpuscles, might play a part in the provision of carbon dioxide for assimilation, because the observed rates of assimilation are many times greater than can be accounted for if carbon dioxide must be changed to carbonic acid to be used in photosynthesis. He assumed the transport of carbon dioxide to the plastid to be effected solely by a concentration gradient. Undoubtedly this plays an important part in carbon dioxide provision, but, according to Arens, carbonates as well as carbonic acid may be taken up by the leaves of water plants against a concentration gradient, and the effectiveness of the leaf in absorbing carbon dioxide indicates the presence of substances which take it up with far greater avidity than a water surface. Whether the photosynthetic apparatus of the plant requires carbonic acid or some other form of carbon dioxide is still uncertain. Burr was unable to find carbonic anhydrase in any of the plants he examined.

Miller & Burr (68) have made experiments indicating that the

lowest carbon dioxide concentration which can be maintained by entire plants (including roots and soil) carrying on assimilation is about 0.01 per cent. Since this was the minimum obtainable over a temperature range from 4° to 37° C., they concluded that photosynthesis and respiration must have the same temperature coefficient. Owing to the inclusion of gas exchange from roots and soil in their experiments, definite conclusions regarding photosynthesis cannot be drawn. Their results are also marred by a large unexplained carbon dioxide production during illumination which appears in many of the curves, particularly at the higher temperatures.

Experiments of Jaccard & Jaag (53), in which the gas exchange of attached leaves of potted plants was measured exclusive of the exchange of roots and soil, show that an assimilating leaf is capable of removing all the carbon dioxide from the surrounding gas space, and of suppressing the appearance of carbon dioxide from respiration. Here again a number of cases of carbon dioxide evolution in the light were mentioned, but the phenomenon seems to have been less persistent than in the experiments of Miller & Burr.

Solarization is mentioned in connection with the carbon dioxide factor because Emerson (23) has attributed the phenomenon to insufficient carbon dioxide supply. He was unable to find any injury to the photosynthetic apparatus of *Chlorella* cells from high light intensity unless the carbon dioxide supply was deficient. Comparing a few instances from other investigators in which a decline in rate of photosynthesis was attributed to intense illumination with others in which no decline was observed, he pointed out that the cases of falling rate were characterized by inferior provision of carbon dioxide. This does not apply to cases where the high light intensity was also accompanied by overheating. Kurssanow (66, p. 242) for example has mentioned that moderate overheating may result in carbon dioxide production during illumination. In Emerson's experiments the decline in rate of assimilation was not accompanied by destruction of chlorophyll, a factor frequently blamed for the phenomenon of solarization. Possibly the type of injury he was studying in *Chlorella* may not be analogous to solarization in leaves, but there are evidently some features in common. Holman (51) for instance found that high concentrations of carbon dioxide retarded or sometimes prevented solarization in leaves.

Dhar's (20) interpretation of solarization as a result of accumulation of end products is improbable in view of Holman's results. His

concept of the entire process, particularly his interpretation of the Blackman reaction, is at variance with simple experimental facts.

MISCELLANEOUS PAPERS

Kohn (63) found that the iodoacetyl radical inhibits the Blackman reaction in *Chlorella*. The nature of the inhibition is uncertain.

An extensive investigation of the effect of hydrogen sulfide on various unicellular algae has been made by Pop (80).

The influence of certain culture conditions on the subsequent rate of photosynthesis has been investigated by Gassner & Goeze. They grew wheat, rye, and oats in cultures containing various amounts of potassium and nitrogen, and exposed to various intensities of light (46, 47); they also tried the effect of different temperatures (48). All these culture factors were found to influence the subsequent rate of photosynthesis, as well as related processes.

Harder, Döring & Simonis (50) have investigated the course of photosynthesis in samples of *Elodea* grown in red, blue, and white light. They conclude that the organism can adapt itself especially to these colors, because each type of material was found to assimilate relatively better in light of the same color in which it was grown (except for white-light material, which did best in red light). The intensities required to give equal rates of photosynthesis in the various colors were compared. The authors conclude that since the amount of chlorophyll plays no direct part in rate of assimilation, the differences in the various types of material must be due either to differences in the carotenoid pigments or to changes in plasmatic factors. It is undoubtedly true that exposure of plants to different wavelengths of light results in changes in the photosynthetic mechanism, but the nature of these changes is not clarified by the experiments in question, because no account is taken of the differences in degree of light saturation represented by the unit rate of photosynthesis in the different samples. Emerson & Green (26, p. 838), in experiments with *Ulva* and *Gigartina*, have shown how misleading conclusions can be drawn from comparisons of relative yields in different colors of light, unless the degree of light saturation is known. Their experiments confirm the common belief that red algae assimilate relatively better in blue light than do green algae, using the rate in red light as a standard of comparison. Their intensity curves, however, show that this may

have nothing to do with differences in the color of the light, and probably depends entirely on differences in degree of light saturation. Since light-intensity curves are wanting in the experiments of Harder, Döring & Simonis, it is impossible to interpret their results in different colors of light.

Burns (17) has made measurements to determine the spectral limits of photosynthesis for white pine and Norway spruce. Peculiarities of the method obscure the significance of the results.

Barker (11), who studied photosynthesis in the marine diatom *Nitzschia closterium* (as well as with a fresh water form, *N. palea*), used 3 per cent sodium chloride containing carbonate and bicarbonate. Such a medium is definitely unphysiological, but may have been satisfactory for his purposes. From a study of respiratory and photosynthetic quotients, which he found to be close to unity, he concluded fat could play only a minor part in the metabolism of these diatoms. As Barker has indicated, a closer study of diatom photosynthesis should be of interest particularly because of their important differences from the green plants, from which most of our knowledge has been gathered.

Gabrielsen (33) reports photosynthesis measurements with cucumbers grown in a greenhouse by daylight supplemented with artificial light. He found high rates of photosynthesis such as are characteristic of sun plants, but intensity curves more similar to those of shade plants.

Some attempts have been made to use heavy water as a tool in the study of assimilation. Shibata & Watanabe (84) found small inhibitions of photosynthesis with 86 per cent heavy water. Curry & Trelease (19) found inhibitions up to 60 per cent with 97 per cent heavy water. Species of *Chlorella* were used in both these investigations. This work is in preliminary stages, and its bearing on photosynthesis is uncertain. Whether the effects are caused by the heavy water itself or by some substance regularly present as an impurity, remains open to doubt. A 60 per cent inhibition of photosynthesis should result in considerable depression of growth, but Meyer (67) succeeded in growing normal cultures of *Chlorella vulgaris* in 96 per cent heavy water, which he had purified by redistillation.

Ficaria verna, a spring-flowering plant which completes its vegetative cycle in the cool weather before the first of June, has been studied in some detail by Mudrack (72), who found a number of interesting adjustments in the photosynthetic mechanism. Assimilation was in-

vestigated by a half-leaf method. Low temperatures and strong light were most favorable to increase in dry weight.

The use of aërial fertilization with carbon dioxide has recently been investigated by Johnston (56). While he finds considerable improvement in yield under laboratory conditions, he believes that the commercial use of aërial fertilization is still far from practical.

Fleischer (28) has extended earlier work on the dependence of assimilation on chlorophyll content, using new methods of controlling chlorophyll content. He finds that magnesium has a specific effect on photosynthesis, besides its influence on chlorophyll content.

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MINERAL NUTRITION OF PLANTS*

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The range of subjects covered by the title is so great that all aspects cannot be dealt with in the space allotted. Reference may be made to several reviews: the absorption of electrolytes by Osterhout (33), the effects of rarer elements by Brenchley (7), and a review by Pirschle (36) which covers the effects of essential elements (potassium, calcium, magnesium, sodium), some of the accessory elements, and the relation of iron to chlorosis. A summary of the literature of ammonium and nitrate nutrition is given by Pardo (35). Attention in this review will be confined mainly to the physiological effects of mineral deficiency. Work on this problem has been proceeding for some years in the laboratory of the reviewer, and as many of the results are still unpublished, salient features of the research are here presented. In so far as recent published work of other investigators deals with this aspect of the problem it is here discussed, though the list may not be complete.

Reference to the work of the author and his collaborators has already been made by Steward (51), and the importance of the method of approach is there stressed. It is clearly desirable to maintain cultural conditions constant so far as external factors are concerned. This is the method employed by Gassner & Goeze (15, 16) and is highly commended by Steward. This method, namely, of maintaining all factors at constant level with the exception of the one studied, is capable of yielding precise information, but is necessarily limited to the actual conditions of the experiment. As the aim of all investigations is to make generalisations of wide application, the consequences of change in one factor should be studied at many levels of the other factors; in a word, the interaction of factors is of equal importance to the study of single factors in isolation. It is of course necessary to employ statistical methods for evaluation of the significance of the single factors and their interactions, but the time is past for putting forward results without statistical estimates of significance. The results of Gassner & Goeze establish clearly enough the importance of interactions, but the adverse criticism by these

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authors of the work of the reviewer and his collaborators on the grounds that interactions were not studied was premature.

Method of study.—A pure line barley (Plumage Archer) has been used throughout the work, grown in sand culture in the open. In the preliminary work (21) one deficiency level of nitrogen, phosphorus, and potassium was studied, but the work was immediately extended to cover a wider range. The standard manuring used maintains excellent growth and gives high yields. The relative proportions of nitrogen, phosphorus, and potassium are not arbitrarily fixed but correspond roughly with the proportions of these nutrients in the grain of barley. The proportions of N : K₂O : P₂O₅ are maintained at 3 : 2 : 1. Five levels of deficiency for each nutrient have been used, each member of a series receiving one-third that of the preceding. Thus the range of concentrations for nitrogen are as follows (per pot of three plants) : N₁, 500 mg ; N₂, 167 mg ; N₃, 56 mg ; N₄, 19 mg ; N₅, 6 mg. For K₂O and P₂O₅ two-thirds and one-third, respectively, are given. The suffixes therefore correspond with increasing deficiencies of nutrient.

In interaction experiments twenty-five combinations have been used. Thus a complete interaction experiment with nitrogen and potassium may be represented as follows :

N ₁ K ₁ (1:1)	N ₁ K ₂ (1:1/3)	N ₁ K ₃ (1:1/9)	N ₁ K ₄ (1:1/27)	N ₁ K ₅ (1:1/81)
N ₂ K ₁ (1/3:1)	N ₂ K ₂ (1/3:1/3)	N ₂ K ₃	N ₂ K ₄	N ₂ K ₅
N ₃ K ₁ (1/9:1)	N ₃ K ₂	N ₃ K ₃ (1/9:1/9)	N ₃ K ₄	N ₃ K ₅
N ₄ K ₁ (1/27:1)	N ₄ K ₂	N ₄ K ₃	N ₄ K ₄ (1/27:1/27)	N ₄ K ₅
N ₅ K ₁ (1/81:1)	N ₅ K ₂	N ₅ K ₃	N ₅ K ₄	N ₅ K ₅ (1/81:1/81)

Each edge of a square, defined by N₁K₁, N₁K₅, N₅K₅, and N₅K₁, as the corners, would represent a series of increasing concentration of one nutrient (limiting series), all other nutrients being present at constant level. A diagonal would represent a simultaneous increase in both nutrients (balanced series). The numerals in parentheses (shown above) represent the relative proportions of the nutrients in terms of the standard amounts given.

General considerations.—To assess the effects of nutrients on growth, efforts should be made to express such relations in numerical terms. The characteristics of growth chosen should throw light on some fundamental physiological process and furnish data capable of further analysis. The characters chosen are: (a) the rate of production and total number of tillers throughout the life cycle, which is a direct measure of meristematic activity; (b) the rate of production

of leaves, which is a measure of differentiation; (c) the growth of the leaf surface, which by further analysis allows the net assimilation rate to be estimated; and (d) the rate of increase and final dry weight of the whole plant and its organs. The plants are sampled at various intervals during growth and the material obtained is analysed chemically to ascertain the amounts and rates of uptake of the nutrients. By this means numerical records of development are obtained. Further analysis of such data is dealt with by Gregory (17). A study of the interaction of water and nitrogen on these lines has been published by Crowther (10). Recently two papers on physiological ontogeny have appeared by Ballard & Petrie (5), and Williams (57), which deal with nitrogen nutrition of wheat and phosphorus nutrition of oats. A full study with barley of the effects on growth of nitrogen and potassium and their interactions has been made by Mathur (29) and of the interaction of phosphorus and potassium by Verma (53) using twenty-five combinations of the nutrients as described above. Some typical results are given below (see also Tables I to IV).

TABLE I

MAXIMUM LEAF AREA PER PLANT IN SQ. CM., 1931 [MATHUR (29)]

	K ₁	K ₂	K ₃	K ₄	K ₅
N ₁	2358	1518	1056	736	716
N ₂	724	699
N ₃	252	...	183
N ₄	107	84	...
N ₅	69	59

TABLE II

MAXIMUM TILLER NUMBER PER PLANT, 1931 [MATHUR (29)]

	K ₁	K ₂	K ₃	K ₄	K ₅
N ₁	12.8	12.9	13.2	14.6	12.5
N ₂	8.9	8.4
N ₃	4.8	...	5.0
N ₄	3.1	2.6	...
N ₅	1.7	1.7

TABLE III

MAXIMUM LEAF AREA PER PLANT IN SQ. CM., 1933 [VERMA (53)]

	K ₁	K ₂	K ₃	K ₄	K ₅
P ₁	1524	...	925
P ₂	910	685	713	528	...
P ₃	507	401	329	315	331
P ₄	307	180	194	130	...
P ₅	258	...	72	...	65

TABLE IV

MAXIMUM TILLER NUMBER PER PLANT, 1933 [VERMA (53)]

	K ₁	K ₂	K ₃	K ₄	K ₅
P ₁	20.0	21.2	24.7	24.0	14.2
P ₂	14.4	14.8	17.4	13.2	9.5
P ₃	10.8	9.3	8.8	8.3	7.8
P ₄	5.8	5.1	4.8	3.9	3.6
P ₅	2.6	2.5	2.5	1.9	2.0

Tiller number and leaf area.—The following results are evident: the effect of deficiency in reducing both tiller number and leaf area occurs in the order $N > P > K$; a decrease in potassium with high levels of nitrogen and phosphorus leads to an increase in tiller number, until the level of potassium is very much reduced; at a low phosphorus level the effect of potassium deficiency on tiller number is completely masked, and, on leaf area, it is much less evident; nitrogen deficiency, in turn, masks completely the effects of phosphorus deficiency; potassium deficiency, unless very acute, has little effect on meristematic activity, and, as is shown later, there is evidence that potassium deficiency does not inhibit protein synthesis.

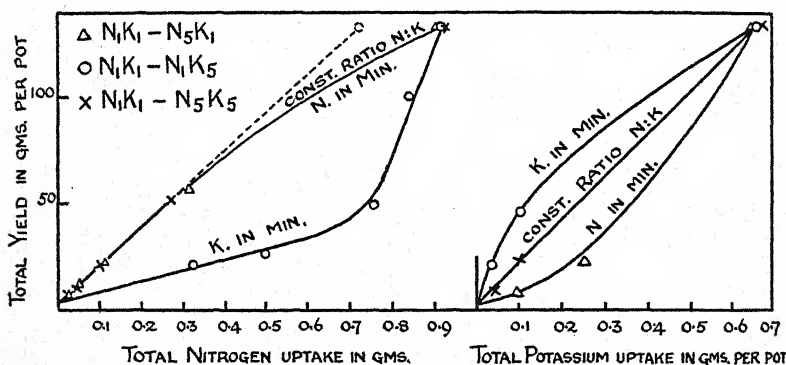


FIG. 1.—Relation between total yield and amounts of nitrogen and potassium taken up. One nutrient in minimum means other nutrient in excess.

Yield.—The relation of yield (total dry weight) to the amount of nitrogen and potassium absorbed is shown in Figure 1. In the case of nitrogen, both where nitrogen is minimal ($N_1K_1 - N_5K_1$) and where nutrients are given in a constant ratio ($N_1K_1 - N_5K_5$), the relation of yield to nutrient absorbed is linear (law of minimum).

Yield plotted against potassium absorbed shows the same relation when both nutrients are balanced ($N_1K_1 - N_5K_5$), but when potassium is minimal ($N_1K_1 - N_1K_5$) the typical Mitscherlich relation appears. In both cases, when yield is plotted against the amount absorbed of the nutrient in excess, a curve of yield with increasing slope is obtained. Chemical analysis of the plants elucidates these relations.

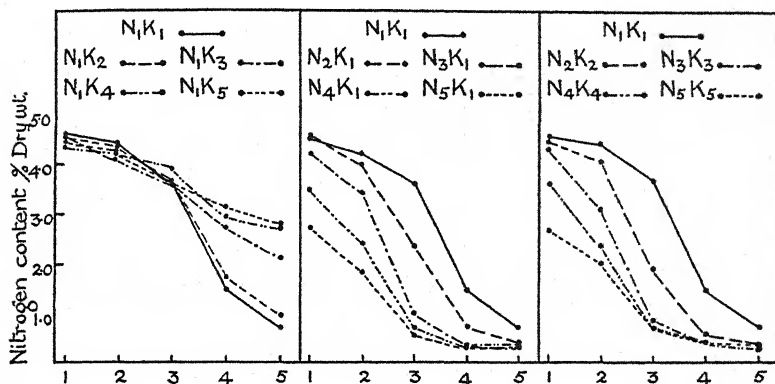


FIG. 2.—Nitrogen content of barley plants sampled at fortnightly intervals. On left, series with potassium in minimum, nitrogen in excess; in center, nitrogen in minimum, potassium in excess; on right, balanced series. (Abscissa represents number of sample.)

For nitrogen they are shown in Figure 2. The series with nitrogen in minimum ($N_1K_1 - N_5K_1$), as well as the balanced series, shows that in the early stages of growth large differences in nitrogen content are apparent, the higher internal concentration corresponding with the higher external concentration. During the life cycle the internal concentration falls and, eventually, in all series reaches almost the same low level. This is in marked contrast to the relation in the series with potassium in minimum and nitrogen in excess ($N_1K_1 - N_1K_5$). Here, in the early stages, all the series show the same nitrogen content in the tissues, but as development proceeds the series receiving the least potassium now shows the highest nitrogen content. Evidently the amount of growth made is the determining factor, the larger the final size the lower the final internal concentration of nitrogen. Similar relations hold for interactions of nitrogen and phosphorus, as well as phosphorus and potassium. This explains fully the so-called "luxury consumption" of nutrients present in excess.

The results will vary, however, as between nitrogen, phosphorus, and potassium owing to their different relations to meristematic growth. Where nitrogen is in excess, and also to a less extent in the case of phosphorus, growth occurs, but excess of potassium does not have this effect. If, in Figure 1 at a given amount of nitrogen absorbed, a line is drawn parallel to the ordinate, it is seen that the yield increases with increase in potassium supply up to the "balanced" dosage, but further increase in potassium leads to no further increase in yield. If the same is done for potassium uptake, the yield increases beyond the balanced dose as nitrogen supply increases. As shown in Figure 2 the lower the level of potassium the higher the final concentration of nitrogen when the supply of nitrogen is in excess of the balanced dose, and this accumulation of nitrogen leads to the excessive tillering noted above. Such plants have a very low assimilation rate, the stems fail to elongate, and the ears although differentiated fail to emerge. Such plants are therefore completely sterile and the nitrogen taken up is not efficiently used. The Mitscherlich curve, therefore, expresses an interaction effect between the nutrient in minimum and that in excess: where nutrients are supplied in balanced proportions the law of the minimum holds.

Balance of nutrients.—This question of salt balance is of very obvious importance. One of the inherent difficulties in evaluation of the effects of any nutrient salt is that the ions are always introduced in pairs, and therefore the combined effect of anion and cation is always obtained. The method of three salt solutions introduced by Shive sought to establish the most effective combination of six ions. In order to evaluate the nutrient effect of single ions from such results a statistical method alone is possible. Beckenbach, Wadleigh & Shive (6) have published such an analysis for corn grown in a series of six salt solutions. They establish the preponderant effect of NO_3^- , and the almost negligible effect of $\text{PO}_4^{=}$ and SO_4^- . The cations, K^+ and Ca^{++} , gave increases in yield throughout the range studied. These results are in complete agreement with a previous analysis of data for potatoes reported by the reviewer (18).

Relation of growth to concentration of nutrients.—The early stages in development are concerned mainly with the accumulation of nutrients which proceeds rapidly in the tissues (Fig. 2). The rate of uptake for all nutrients studied has been found to be proportional to external concentration up to the stage at which floral differentiation begins (six weeks in barley sown in May). The maximum rates

of uptake of nitrogen and phosphorus are shown in Figure 3. From germination onwards the rate of uptake increases as the root system expands. Two opposing processes regulate the rate: with expansion of the root system the rate tends to rise and, as uptake increases, the external concentration falls by depletion of the solution. As a result, therefore, the rate of uptake reaches a maximum value early in the life history. Since growth is an exponential process with an ever-increasing number of primordia laid down as new leaves and tillers,

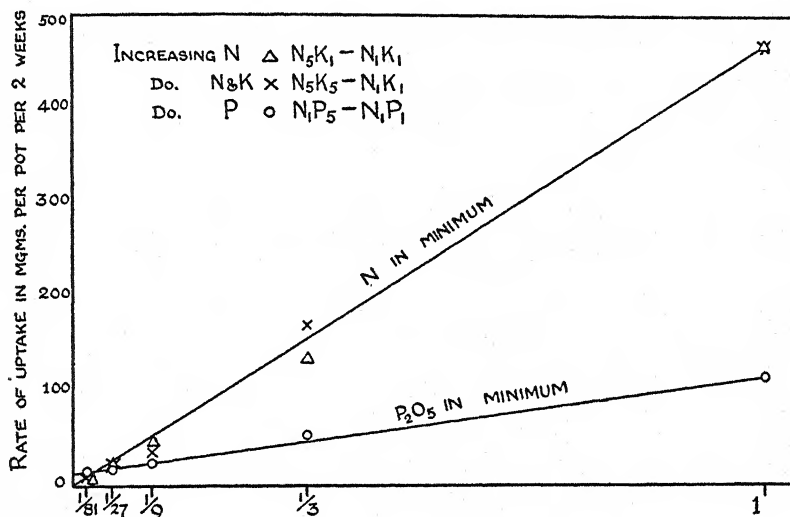


FIG. 3.—Relation between rate of uptake of nutrients and external concentration; experiments with barley six weeks from germination. (External concentrations of N and P₂O₅ are represented on the abscissa.)

the requirement of nutrients for further growth and maintenance tends also to an exponential increase; meanwhile, the external solution is depleted and, therefore, the rate of supply falls. The critical point at which demand overtakes supply marks the maximum in rate of uptake. This point coincides with the appearance of deficiency symptoms in the leaves at emergence, and has been called the stage of "internal starvation" (10). Up to this stage leaf-area growth and dry-weight increase are exponential (17). Where high levels of nutrient are presented at the outset, or where nutrient supply is continuously renewed, internal starvation supervenes nevertheless. This has been shown in barley to be due to a sudden fall in the rate of up-

take by the roots. A similar cessation of uptake was found in cotton by Crowther (10), and is related in both cases to the onset of the reproductive phase.

The rate of development is proportional to the rate of uptake and therefore to external concentration. For each concentration, therefore, if not too high, the rate of uptake and rate of depletion are proportional to external concentration. Maxima in tiller number and leaf area should therefore occur at the same time independently of the concentration at which the plant is grown, and this has been shown to be the case with phosphorus and potassium in minimum.

The onset of internal starvation has an obvious bearing on the optimal time at which nutrients should be supplied, and on recovery from starvation. Tibeau (52) has reported on the time factor in the utilisation of nutrients by hemp, and shows that recovery from prolonged starvation of nitrogen is very slow. An interesting relation to sex differentiation is stressed, low nitrogen producing male flowers and high nitrogen producing female flowers.

A comparative study of rye and oats in relation to mineral nutrition has been made by Deleano & Gotterbarm (14). Losses of potassium, calcium, and nitrogen occur after flowering in rye, whereas oats lose potassium only. The concentration of the sap is maintained constant, when desiccation after flowering begins, by excretion of salts by the roots.

Differential varietal response.—Reference may here be made to the efficiency with which different varieties utilise nutrients in yield production. This so-called "differential varietal response" was first established with five varieties of barley by Gregory & Crowther (19, 20). Lynes (27) has examined the phosphorus nutrition of twenty-one varieties of corn and has noted different symptoms of deficiency and different rates of utilisation in the early stages. Large differences in susceptibility to phosphorus deficiency were noted among the varieties. A high phosphorus requirement acted as a recessive in hybrids. The response to phosphorus is correlated with the number and character of the roots.

Lamb & Salter (26) established differential response by statistical analysis of yield with seventeen varieties of wheat grown at different levels of fertility. Differential response in wheat has been shown also by Woodford & McCalla (58). Crowther and Crowther, Tomforde & Mahmoud (11, 12) in large-scale field experiments have established the same for varieties of cotton.

Symptoms of deficiency.—There is general agreement as to the symptoms of nitrogen and phosphorus starvation. The symptoms of potassium starvation, however, have been variously described and are discussed by Richards & Templeman (39). These may be either a light yellow colour of leaves associated with succulence and very rapid death of the leaves, or a dark green colour associated with white or brown spots on the leaves. The first type is characteristic of potassium starvation with the standard manuring used in these experiments, in which a high level of sodium is maintained. An additional effect under such conditions of low potassium and high sodium is the continued tillering which occurs after eight weeks, when, with ample supply of potassium, tillering ceases. The first crop of tillers dies and is succeeded by a second and third cycle which in turn die without emergence of ears. In the second type described by American investigators (see 39 for references) meristematic activity ceases and growth rate falls. This second type of potassium deficiency is characteristic of conditions of high calcium and low phosphorus supply. Shih (49) has shown that even in the presence of high calcium the first type of deficiency symptoms is produced by adding extra sodium. Sodium, therefore, has a specific effect on growth. Scharrer & Schropp (44) find no relation between sodium and the uptake of other nutrients, but state that increasing concentration of sodium leads to greater uptake of potassium as well as sodium. Shih on the other hand finds that a decrease in potassium in all series studied leads to an increase in sodium uptake.

Schneider (46) has examined the effects of deficiencies on the anatomical structure of *Pelargonium*, and the effects on the protoplasmic structure of the leaves of *Elodea* are described by Kalchhofer (25).

Water content.—Results in these experiments, discrepant from those of American investigators, have been consistently obtained; thus potassium deficiency of the first type (high sodium, low calcium) is associated with increased succulence, and that of the second type (high calcium, low phosphorus) with low water content. The work of Shih (49) has shown that the water content with potassium deficiency depends on the levels of sodium, calcium, and phosphorus. The relation of water content to relative concentrations of sodium, calcium, and potassium, as found by chemical analyses of green leaves in barley, are represented in Figure 4 in which the contours are of equal water content (water/dry weight). The manurial combinations

used are indicated below the diagram. The following results have been shown to be statistically very significant: (a) Phosphorus content has little effect on water content at a high potassium level, but an increase in phosphorus deficiency reduces the water content as the potassium level falls, i.e., reduction of potassium increases the water content

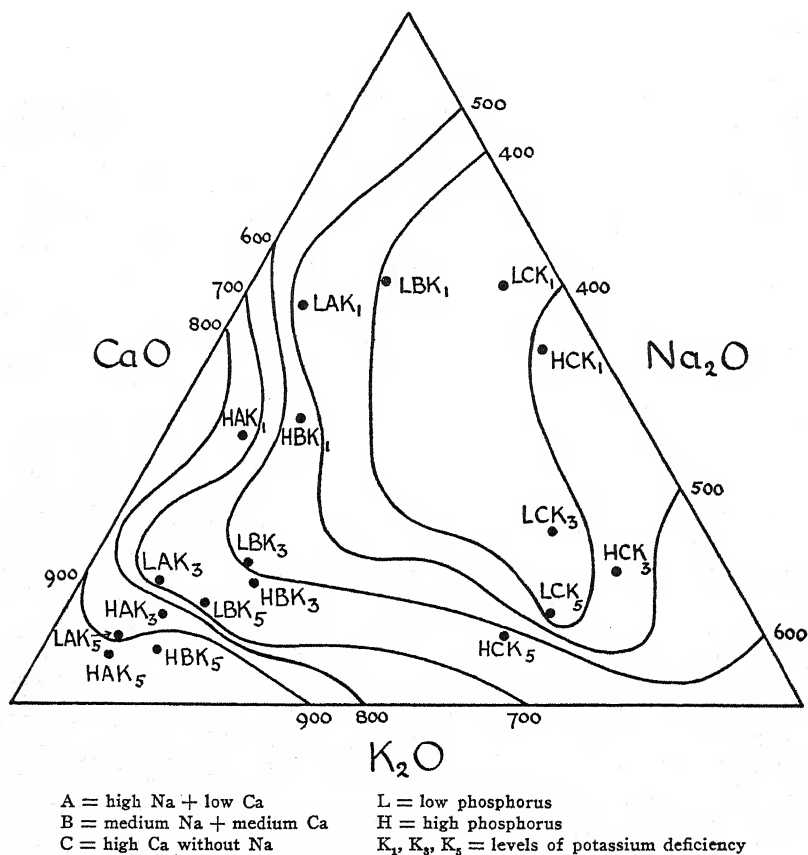


FIG. 4.—Relation between water content and relative proportions of cations in green leaves of barley. Contours are of equal water content.

much more at high than at low phosphorus levels. (b) Reduction in water content due to a phosphorus deficiency is much more evident at high sodium than at high calcium levels. (c) At a high sodium level, potassium deficiency leads to an increase in water content; in balanced

sodium-calcium series the effect of potassium is less marked; in a high calcium series, reduction of the potassium level leads to reduction in water content, but a further reduction in potassium leads to a second increase. These results completely clear up the discrepancies in the findings of different investigators.

Nitrogen metabolism.—A detailed study of the various nitrogen fractions in the tomato has been made by Clark (9) on plants fed with nitrate and ammonia. The methods for study of nitrogen metabolism are dealt with by Orcutt & Wilson (34). The so-called reductase activity of expressed sap has been reinvestigated by Sommer (50) who fails to find evidence for catalytic reduction of nitrate to nitrite in absence of light.

A study of deficiencies in nitrogen, phosphorus, and potassium (1/10 standard) on the nitrogen metabolism of barley leaves has been published by Richards & Templeman (39). The successive leaves of the main shoot at full emergence and during senescence were studied. It should be noted that the number of successive leaves is almost independent of manuring, ranging from ten to twelve in extreme cases. The following fractions were estimated: total nitrogen, protein, total amino, amide, nitrate, and residual crystalloid nitrogen. In general, total nitrogen and most of the fractions reach a maximum in the second to fourth leaf, decline to a minimum in the eighth or ninth leaf and again rise in the last leaves. Two sources of nitrogen are available: newly absorbed nitrogen and nitrogen liberated by proteolysis. A study of the conditions leading to protein breakdown in relation to chlorophyll destruction has been published by Michael (30).

Nitrogen deficiency leads to low content in the leaves, but the fractions are present in the same proportions as in fully manured plants, indicating a normal course of protein synthesis. Phosphorus deficiency leads to reduction in protein content even at emergence, and a rapid fall during senescence of individual leaves. The characteristic effect is the accumulation of amide nitrogen and a less marked accumulation of amino nitrogen and nitrate in the later leaves. A check in protein synthesis at the level of amide is indicated, and this failure of protein synthesis leads in the case of phosphorus, as in nitrogen deficiency, to a low meristematic activity as shown by reduced tiller production and individual leaf size and leaf area. Potassium deficiency (with high sodium, low calcium) is characterised by: (a) a marked increase in amino and amide nitrogen, though they appear in normal proportions; (b) accumulation of nitrate nitrogen in later leaves;

(c) a very rapid breakdown of protein during senescence of leaves. At emergence the leaves show a normal protein content. It is held that potassium is not primarily associated with protein synthesis but is necessary for maintaining the protoplasmic complex, and in its absence rapid proteolysis occurs. In consequence of this protein breakdown, soluble nitrogen fractions accumulate throughout the plant. The effects of high calcium and low sodium in modifying these conclusions are not yet known.

Carbohydrate metabolism.—The effect of nitrogen supply on sugar production in sugar cane has been studied by Das (13). High nitrogen supply increases succulence, and the variations in water content are related to different absorption of inorganic salts. Hydration is correlated with growth and the content of reducing sugar and sucrose. The sucrose content of expressed sap is highest with nitrogen deficiency, but no relation to polysaccharides is found. It is concluded that the inorganic constituents, acting on the enzymes, are the chief controlling factors.

A study of deficiencies in nitrogen, phosphorus, and potassium (1/10 standard) on the leaves of barley has been made by Gregory & Baptiste (22) using successive leaves on the main shoot, both at emergence and during senescence. The experiment was repeated in two years with similar results. The standard manuring with high sodium and low calcium was used.

Nitrogen deficiency has no consistent effect on reducing sugar, while total carbohydrate is greatly increased. Phosphorus deficiency increases free reducing sugar, but total sugar is less affected. Potassium deficiency lowers reducing sugar, and leads to a very low total sugar content. No consistent differences in sugar content appear in the deficient series until after the emergence of the fourth leaf, at which time tillering begins. A minimum sugar content occurs at this time. Except in the potassium-deficient series the sugar level rises from this point throughout the major part of the life cycle. The course of this rise depends on the mode of application of the manures.

Senescence in the earlier leaves leads to a fall in sugar content but in the later leaves the opposite effect appears, the change-over occurring in different leaves in the various series. Potassium deficiency alone shows, in all cases, a consistent fall in sugar content during senescence. Characteristic differences in the ratio of sucrose to free reducing sugar are found: very high values in nitrogen deficiency, very low in potassium deficiency, and intermediate in phosphorus

deficiency. All the effects noted were highly significant statistically. The high value of reducing sugar in the phosphorus-deficient leaves is shown to be related to the high ratio of amide to total amino nitrogen in this series, and a sudden increase in both values occurs at the time of emergence of the fourth leaf. The sugar level is related to carbon assimilation, translocation, protein synthesis, and respiration. High sugar content with nitrogen deficiency is due to high assimilation rate, low respiration, low protein synthesis, and low meristematic activity. Lower assimilation rate with phosphorus deficiency is offset by the low meristematic activity so that the sugar content does not depart much from normal. In the potassium-deficient plant the very low assimilation, high respiration, active protein synthesis, and excessive meristematic activity all contribute to a very low sugar content.

The investigations of the effect of potassium deficiency on the barley leaf have been extended to cover conditions of high calcium and low sodium. Three levels of potassium (K_1 , K_3 , K_5) combined with two levels of phosphorus have been used. A fructose anhydride has recently been isolated from water extracts of barley leaves by Archbold & Barter (1) and estimates of this carbohydrate fraction were included in this experiment.

Some of the results obtained are entered in Table V. A key to the manurial combinations is given. The values entered are means for the ten successive leaves, expressed as equivalent hexose in milligrams per gram of leaves (fresh weight).

The following results appear: (a) The total carbohydrate content is greater, on the average, in the series with high calcium than with high sodium; (b) potassium deficiency leads to a great reduction in all fractions in the presence of high sodium, but in the series with high calcium reduction in carbohydrate is not at all evident at K_3 level and is only very small at K_5 level; (c) reduced phosphorus in the high sodium series alone leads to an accumulation of reducing sugar and fructosan. Fructosan accumulation is also dependent on the potassium level, low potassium leading to a fall in fructose content.

Effect of nutrient deficiency on respiration.—The literature on the effect of nutrient deficiency on respiration and assimilation is very limited. Much of the previous work suffers from the following defects: (a) insufficient regard has been paid to the changes in rate which take place during development; (b) evaluation of statistical significance is seldom attempted.

Estimations have been made of respiration, etc., of successive

leaves on the main stem at the time of complete emergence by Gregory & Richards (21), and Richards (37). The use of observations made on all leaves simultaneously has been avoided for the reasons given by Richards (38) who points out that this method confounds the differences of metabolic rate in leaves formed at different stages in the life history of the plant with the senescent changes in individual leaves; the metabolic history of each leaf varies and due regard must be paid to this fact. Only results confirmed by statistical analysis are accepted.

TABLE V
CARBOHYDRATE FRACTIONS AS EQUIVALENT HEXOSE
(mg. per gm. fresh weight)

	Reducing Sugar	Sucrose	Fructosan	Total Sugar
HAK ₁	2.4	16.3	5.9	24.7
HAK ₃	2.7	14.9	1.7	19.4
HAK ₅	1.9	7.3	1.1	10.2
LAK ₁	4.3	16.7	11.3	32.3
LAK ₃	3.5	16.4	4.4	24.3
LAK ₅	2.8	8.4	3.7	15.0
HCK ₁	4.5	17.3	5.4	27.2
HCK ₃	4.7	16.4	3.4	24.5
HCK ₅	4.1	18.9	2.6	25.8
LCK ₁	4.6	18.5	9.6	32.6
LCK ₃	4.6	19.9	9.3	33.9
LCK ₅	5.3	15.4	5.2	26.0

H = high phosphorus

A = high sodium

L = low phosphorus (1/5)

C = high calcium

K₁, K₃, K₅ = levels of potassium (1, 1/9, 1/81)

The relation of respiration to water content is discussed by Schlieper (45).

Nitrogen deficiency in barley always leads to a reduction in the rate of respiration (21, 48). This agrees with the findings of Müller & Larsen (32), Ruhland & Ullrich (41), and Hamner (23).

The respiration of successive leaves within a series shows an increase at first and reaches a maximum at the second to fourth leaf; it declines to a minimum at the eighth or ninth leaf and rises subsequently. This agrees with the changes in total nitrogen already men-

tioned (39). Phosphorus deficiency at the level 1/5 results in no significant reduction in respiration (21), but on further reduction of the phosphorus supply a progressive decrease in respiration results.

Jones (24) has investigated the effect of phosphorus supply on etiolated wheat seedlings and has shown a small increase in carbon dioxide production with addition of phosphorus. He concludes that phosphorus acts by its association with carbohydrate metabolism and by its regulation of protein synthesis.

Potassium deficiency at the level $K_3(1/9)$ leads to a very great increase in respiration but further reduction in potassium leads to a fall in carbon dioxide production, which in extreme starvation is below normal (37). These results were obtained with high sodium and low calcium, i.e., pale green succulent leaves.

Respiration of leaves under conditions of high calcium, i.e., dark green leaves with low water content has since been studied. Again great increases in respiration are found with potassium deficiency; the mean respiration rate of successive leaves in the high calcium series at two levels of phosphorus are given in Table VI.

TABLE VI

RESPIRATION RATE; MEAN OF ALL SUCCESSIVE LEAVES
(mg. CO_2 per gm. dry weight per hour)

HCK ₁	4.38	LCK ₁	4.70
HCK ₃	5.42	LCK ₃	4.60
HCK ₅	6.51	LCK ₅	4.96

The increase in respiration with potassium deficiency is thus found to be general in barley. The effect of potassium is dependent, however, on the level of phosphorus; the effect of potassium deficiency is masked when phosphorus is kept at a low level.

The relation of respiration to carbohydrate and nitrogen metabolism has also been investigated. Sen (48) studied the relations in series of minimal nitrogen and potassium

$$(N_1K_1 - N_5K_1; N_1K_1 - N_1K_5).$$

Estimations of protein, amino nitrogen, reducing sugar, and total carbohydrate were made. In all series with the single exception of N_1K_5 significant high positive correlations between respiration and protein content were found. As also observed previously (39) potassium deficiency gave a very high amino nitrogen content, and in this

series ($N_1K_1 - N_1K_8$) a significant high correlation of amino nitrogen and respiration was noted. Very high values of sugar in the nitrogen-deficient and very low values in the potassium-deficient series were found (cf. 22). Thus the nitrogen-deficient series had a low respiration associated with high sugar content and the potassium-deficient series, the converse. Indeed in no case within a manurial series was there a significant direct correlation between respiration and either total or reducing sugar content with the single exception of the N_1K_8 series which gave a high positive value with total sugar. The partial correlation, after eliminating amino and protein nitrogen effects, was still very high and significant, and in the N_1K_8 series also a significant positive partial correlation of respiration rate and sugar was obtained when the effect of amino nitrogen was eliminated. It thus appears, as Richards surmised (37), that with very great potassium deficiency carbohydrate becomes the controlling factor in respiration, but in no other case is this so. The very high correlations of respiration with protein (nitrogen deficient) and amino nitrogen (potassium deficient) are unaffected by eliminating the effect of sugar content of the leaf.

A similar study has been made of respiration in the series with minimal phosphorus and also in interaction with potassium (balanced series) in relation to carbohydrate and nitrogen fractions. When potassium is reduced at the lower level of phosphorus from P_3K_1 to P_3K_8 there is again a significant increase in respiration, but at the P_5 level reduction in potassium has no effect. A similar relation to amino nitrogen has been obtained. A striking similarity is thus seen throughout between the effects of potassium deficiency on respiration and amino nitrogen content. Schwabe (47) has obtained striking effects of amino acids on the oxygen uptake of *Elodea* leaves. As a result of feeding with tyrosine and glutamic acid, increases of 300 per cent in respiration were obtained. By periodical removal from amino acid solution to water the effect disappeared, to reappear again on replacing in the amino acid solution. The amino acid was thus used up but the respiratory quotient was unaffected, indicating that the amino acid is not directly oxidised. It is suggested that the amino acids stimulate general oxidation by acting as hydrogen acceptors.

Returning to the experiments with barley it thus appears that the effects of phosphorus deficiency on amino nitrogen accumulation and respiration are opposed, a decrease in respiration rate being accompanied by an increase in amino nitrogen; potassium starvation, on

the other hand, leads to an increase in amino nitrogen as well as in respiration. In the series P_3 and P_5 the correlations of respiration with protein are not significant, but high positive correlations with reducing sugars are found. The effects of phosphorus and potassium deficiencies are thus very different, the effect of the former apparently working through carbohydrate relations, the latter through nitrogen metabolism.

Feeding experiments.—A brief reference may here be made to experiments performed to elucidate the relations mentioned above by feeding leaves from starved plants of barley with the particular nutrient in deficiency. Said (42) worked with potassium-starved leaves and showed that feeding with potassium alone had no effect, which demonstrates that the effect of potassium is indirect. However, feeding with sugar (glucose, fructose, or sucrose) increased the respiration rate.

With phosphorus-starved leaves feeding experiments have been performed by Sankaran (43). Leaves removed at different stages during the life cycle were fed as follows: (a) distilled water, (b) sucrose in excess, (c) sucrose and sodium phosphate, and (d) sucrose, sodium phosphate, and ammonium nitrate. In addition to carbon dioxide production, estimations of phosphorus content, protein nitrogen, amino nitrogen, amide nitrogen, and residual nitrogen were made before and after the period of respiration (three or four days).

It was found that respiration was increased very considerably by addition of phosphorus and sugar, and an additional increase was obtained by giving ammonium nitrate with the sugar. The magnitude of the increase, however, depended upon the stage at which leaves were removed, the effect being greatest in the earlier leaves; thus the effect of feeding with phosphorus was greatest when the leaves normally contained most phosphorus. In fact the effect of phosphate addition on increasing the respiration is greatest when the respiration is normally highest. Schwabe (47) points out a similar effect of amino acids on *Elodea*.

It was also observed that considerable protein hydrolysis occurred during respiration but this was always less in the leaves fed with ammonium nitrate.

The conclusion reached from these experiments, though the evidence cannot be presented here, is that the main effect of phosphorus on respiration depends on its relation to nitrogen metabolism, and the effect on carbohydrate metabolism is secondary. The evidence is not

inconsistent with the view that carbohydrate is not oxidised unless it is first drawn into the cycle of nitrogen metabolism. On this view the effect of potassium deficiency is related to amino nitrogen content because the level of this fraction measures the rate of proteolysis. As at the same time, as has been shown, the protein content of potassium-starved leaves is at first very high, it follows that protein synthesis as well as hydrolysis must be taking place in potassium-starved leaves very rapidly; this, it is suggested, is related to the high respiration rate. Further discussion cannot be pursued here.

White (55), working with *Lemna*, finds during recovery from nitrogen starvation that the carbohydrate which has accumulated during starvation is very rapidly oxidised. The respiration rate may reach a value of five times that previously shown under nitrogen deficiency. Rohde (40) also discusses the effect of potassium on respiration. He suggests that potassium regulates the distribution of iron in the plant; the iron, in turn, acts as a catalyst for oxygen uptake. The increased oxygen supply inhibits anaërobic respiration, and the effect of potassium therefore is related to the relative rates of aërobic and anaërobic respiration.

Effect of nutrient deficiency on carbon assimilation.—Experiments have been performed in which the assimilation rates of barley leaves from plants deficient in nitrogen, phosphorus, and potassium have been studied in full daylight, using air as the source of carbon dioxide. Successive leaves were again used, while attached to the plant. The air supply was very rapid, the assimilation chamber shallow, and the leaf area small so that maximal rates of assimilation were obtained. Nitrogen deficiency at the levels $N_3(1/9)$ and $N_5(1/81)$ were studied by Chinoy (8). No reduction in assimilation rate was found as may be seen from the following values:

Assimilation of Carbon Dioxide (mg. per sq. decm. per hour)			
Control, fully manured.....	N_1	19.4	} Mean value of 17 determinations
Medium deficiency	$N_3(1/9)$	20.1	
Control, fully manured	N_1	20.1	} Mean value of 11 determinations
Extreme deficiency	$N_5(1/81)$	20.1	

This confirms the previous findings of Gregory & Richards (21). Similar experiments with phosphorus and potassium-deficient leaves show, after the fourth leaf, a rapid fall in assimilation which reaches a minimum in the eighth leaf and subsequently rises (cf. 21).

The results with nitrogen deficiency are not in agreement with those of Müller (31), Müller & Larsen (32), and Hamner (23), who record a reduction in assimilation rate due to nitrogen deficiency. This discrepancy is in part due to the methods used by these investigators: no effort was made to separate the senescent effect of lack of nitrogen on individual leaves from the effect on the leaf at the time of its emergence. There is no doubt that nitrogen deficiency leads to a more rapid senescence of leaves and this is even more evident with phosphorus and potassium deficiencies. Unless these factors are separated discordant results must be expected. Gassner & Goeze (15, 16) have stressed the importance of the interactions of nitrogen and potassium on assimilation rate. In their experiments the first leaf alone was used, which, as Steward (51) has rightly pointed out, would be least likely to suffer from nutrient deficiency owing to stores in the seed. In our experiments the marked effects on leaves just emerged appear at the fifth leaf, and it is interesting to note that the first four leaves of barley are preformed in the embryo, the fifth being differentiated during germination. It would thus appear that the conditions under which the primordia are laid down are very important in determining the reaction to nutrient supply. In the experiments of Gassner & Goeze the effects on assimilation in the different series become more marked as the experiments proceed (16), and indeed, with leaves ten days old, changes in the nitrogen supply (1/10, 1, and 5) were without effect on the assimilation rate. Senescence was, therefore, primarily investigated in this work. The relation of potassium to dry matter accumulation is discussed by Maiwald & Frank (28).

The results obtained with barley by direct estimations of assimilation are in agreement with estimates of net assimilation derived from sampling data. The method is described by Gregory (17); it is, without doubt, the best method available for studying average assimilation rates over long periods. Data from such estimates are given by Gregory & Baptiste (22). By this method nitrogen deficiency up to maximal leaf area (i.e., until senescence of leaves begins) shows no effect on assimilation [Gregory (17), Mathur (29)]. This has been shown for cotton also by Crowther (10). Verma (53) showed by the same method with barley that phosphorus and potassium deficiencies reduce assimilation in the order stated. Watson (54), from sugar analyses on potato, concludes that addition of potassium chloride increases assimilation rate. This important paper, however, is primarily concerned with diurnal variations in carbohydrate fractions and in

water content. White (55), working with *Lemna*, found no reduction in net assimilation as a consequence of nitrogen starvation, though low respiration, and low multiplication rate were symptomatic. Further, the same investigator finds a low assimilation rate to be characteristic of potassium deficiency (56). During the course of potassium starvation dry matter accumulates in the fronds so that the dry weight per unit area reaches a high constant level. On renewing the potassium supply the accumulated dry matter rapidly disappears. Enzyme extracts show a reduced capacity for starch hydrolysis with potassium deficiency. The main effect of potassium is attributed to the regulation of carbohydrate metabolism by control of the starch-sugar balance.

In conclusion, to indicate some of the complexities of the relations of assimilation to nutrient ions, reference should be made to the important papers of Arens (2, 3, 4) who has investigated the assimilation of aquatic plants and has shown that calcium bicarbonate molecules are absorbed at the lower surface of the leaf while calcium hydroxide escapes at the upper. This polar transport is found only in the light, no absorption occurring in the dark. In the case of potassium bicarbonate absorption, potassium carbonate escapes from the upper surface.

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ORGANIC ACIDS OF PLANTS*

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It is exceptional to find plant tissues, the pH values of which exceed 5.5. In many cases this acidity of the tissue is associated with the presence of oxalic acid, lactic acid, or acids of the malic group together with their salts (21, 22, 23, 24, 25). It is with the metabolism of these acids that this review deals.

The last decade has seen the birth of many hypotheses of plant-acid metabolism often ill-supported by experimental work. Such experimental work has until recently been hampered by the absence of accurate and specific analytical methods for determination of individual plant acids, and a proper evaluation of any particular piece of work is only possible after critical examination of the analytical methods used. Most workers have determined one or more of the following: the titratable acid; the total acid titratable between pH values 2.5 and 8.0 by the Van Slyke-Palmer (50) method, which includes part of the phosphate as well as organic acids; the total acid precipitable by lead acetate, which again includes phosphate and sulphate; or the acid extractable with ether. These "values" clearly have to be interpreted with caution. In a few cases determinations of citric acid by some method based on Stahre's reaction, of *l*-malic acid polarimetrically (2), and of oxalic acid by precipitation of its calcium salt have been carried out. Until 1934 no specific method for the determination of *dl*-malic acid had been devised. The rather crude earlier methods are now superseded, however, by the successful development by Pucher, Vickery, Sherman, Leavenworth & Wake-man (39, 40, 41, 42) of a technique for the determination of *dl*-malic and citric acids which are specific for these two acids. Little work as yet has been published in which these methods have been used, but thanks to these workers it is now possible to determine the three more widely spread plant acids, malic, citric, and oxalic.

Before leaving the subject of analytical methods mention must be made of the many researches carried out in Germany in which use has been made of the analytical processes developed in Leipzig. These processes were in use, apparently, in 1926 but have never been divulged though amazing claims of accuracy are made for them. Bendrat, for example, who used the Leipzig methods, quotes quan-

* Received January 19, 1937.

tities of acid present to four places of decimals. Workers in other laboratories who give details of their methods are prepared to admit errors of 5 per cent (41, 11). A number of inconsistencies appear in the published work emanating from Leipzig which suggest that the accuracy and specificity of these unpublished methods are greatly over-estimated. An example of this is found in analyses by Schwartz (46) whose results show that the ratio, ["free" *l*-malic acid]/["free" *d*-malic acid], in *Pelargonium*, was five times the ratio, [combined *l*-malic acid]/[combined *d*-malic acid], whereas in fact these two ratios must be equal since the dissociation constants of the *l*- and *d*-acids are equal. The claim that the "free" acid can be determined [Schwartz (46)] is also remarkable as the methods already known suffice only to determine total acid, titratable acid, and hydrogen ion concentration. From these data the concentration of free acid can be calculated approximately when the dissociation constants are known (7). A somewhat similar apparent impossibility is found in the results of Wolf (60). He quotes data of quantities of total acid and acid salt in the tissue. A dibasic acid in solution within the pH limits found in living tissues consists of bivalent ions, univalent ions, and undissociated molecules (7). It is not possible to determine the quantity of each species separately in such a solution as a plant sap; the meaning attached by Wolf to the term "concentration of acid salt" is therefore quite obscure, as is the meaning attached to the term "free acid." It is particularly unfortunate that this cloud of uncertainty hangs over the experimental methods of the Leipzig school as the value of the ten papers published by them in recent years is, in consequence, almost impossible to assess.

Equally important with problems of analytical technique are those of sampling which have received very scant attention from workers on plant acids. Many (in fact, most) statements on the effects of various treatments or environmental conditions on acidity of tissues are based on two analyses and no attempt is made to test statistically the significance, if any, of the difference between the two analyses. Obviously results of this type should be treated statistically, or quoted in a form which would make statistical treatment possible.

THE FORMATION AND DISAPPEARANCE OF ORGANIC ACIDS IN PLANT TISSUES

Researches on plant-acid metabolism resolve themselves into enquiries into chemical mechanisms (the sequences of reactions involved

in formation and loss of acids from tissues), and also enquiries into the rôle which these reactions play in the life of the plant. To the biologist this latter field of enquiry is the ultimate aim, and useful contributions to this field are only possible after a much fuller exploration of the chemical mechanisms.

The acid metabolism of relatively few plant types has been investigated. The more important are: a few succulent plants (*Bryophyllum*, *Sedum*, *Crassula*, *Opuntia*, *Kleinia*); tobacco (*Nicotiana*); tomato (*Lycopersicum*); rhubarb (*Rheum*); *Begonia*; and mould fungi (*Aspergillus* and *Penicillium*). Only cursory examination of a few other plants has been carried out with the object of throwing light on their acid metabolism; there is, therefore, little experimental justification for assuming uniformity of acid metabolism in different plant types.

The dogma of uniformity of metabolism is, however, widespread and possibly justified. It is applied rigidly to the much investigated process of alcoholic fermentation, and a belief that sugar breakdown follows that sequence of reactions with eventual formation of pyruvic acid as a key intermediate product has dominated many of the workers on plant-acid metabolism. These workers hold that the plant acids are formed either from pyruvic acid derived from sugar, or that the malic and oxalic acids are formed from protein rather than carbohydrate sources. These workers accept without proof the occurrence in all plants of the reaction sequences associated with the names of either Neuberg or Meyerhof; the most important step in the "proof" of the reaction sequences they advance for formation of malic and other plant acids is therefore derived from investigation of the somewhat abnormal metabolism of yeast.

A small body of workers uphold the view that the plant acids are formed from carbohydrate by reactions quite distinct from those associated with the zymase system. Different workers claim to support these divergent views from investigations on more than one of the plant types examined. It is therefore simplest to classify recent work according to the type of plant examined.

Plants in the tissues of which diurnal periodicity of acidity occurs.—Many plants accumulate acids in their leaves and stems at night and these accumulated acids disappear during the following day. Plants from a great many natural orders show this interesting phenomenon which is often thought to be associated with the "succulent" habit; most succulent plants do show it, so also, however, do

many non-succulents as has been known since 1886 [Warburg (56), Schwartze (46)]. The increase in acidity is generally assumed to be due to formation of malic acid since Franzen & Ostertag (21) showed that the accumulated acid in *Echeveria* (one of the Crasulaceae) was largely malic acid. More recently it has been shown that accumulations of citric acid also occur, in addition to malic acid, in *Kleinia* and *Bryophyllum* [Borgström (14), Guthrie (28, 35)]. This acid, produced during the night, does not remain in the form of free acid but enters into equilibrium with other ionic and molecular species in the sap. A solution of a dibasic or polybasic acid containing relatively small amounts of the free acid together with large amounts of univalent and bivalent ions is buffered in respect to free acid concentration (7). Addition or removal of free acid has very little effect on the concentration of free acid (cf. buffers for pH regulation), as it is largely converted into univalent ions by interaction with bivalent ions and vice versa.

It has been shown [Wolf (60)] that in three species of *Bryophyllum* the nightly increase in acid is not associated with any change in the content of protein and amino acid. There is a marked loss of carbohydrate associated with a gain of acid in *Bryophyllum* (60) and *Sedum* (8). At least one molecule of sugar disappears for each molecule of acid accumulated. Unfortunately some of the difficulties of these sugar analyses have not been realised (8, 60); these difficulties are due largely to the presence of sedoheptose and in certain cases its anhydroderivative sedosan (8, 29, 33, 34, 38). Each of these, when warmed with 1 per cent hydrochloric acid, is converted into an equilibrium mixture containing roughly twenty parts of sedoheptose to eighty parts of sedosan. The latter does not reduce alkaline copper or ferricyanide solutions. The extract of tissue in 80 per cent alcohol includes hexose monosaccharides, sucrose, sedoheptose, and sedosan. The sucrose cannot be determined by inversion with acids since sedoheptose is converted to sedosan by this same treatment; nor can it be assumed without investigation that "invertase" preparations have no effect on sedoheptose. Further, the reducing power of the unfermentable (by yeast) residue represents the sedoheptose content only; sedosan is not included.

According to Bennet-Clark accumulation of malic acid in detached *Sedum* leaves is associated with loss of roughly equimolecular quantities of sedoheptose. When the leaves are attached to the stem these relationships are made more complex by transport of material between

leaf and stem. The formation of malic acid is associated with a negligibly small output of carbon dioxide; thus some 6 to 8 mg. equivalents of acid are formed with a corresponding output of about 1 millimol of carbon dioxide (8, 60).

Wetzel & Ruhland (59) and Wolf (60) assume that the malic acid produced must arise from pyruvic acid because they assume that the Neuberg reactions must account for sugar breakdown. Conversion of pyruvic to malic acid might proceed thus:

-CO₂

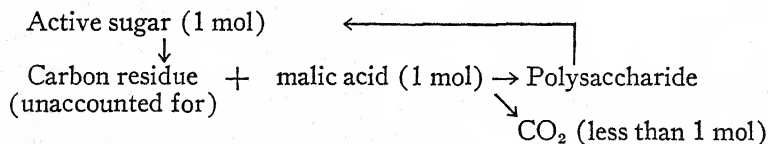
Pyruvic acid $\xrightarrow{\quad}$ acetaldehyde \rightarrow acetic acid \rightarrow malic acid
or pyruvic acid \rightarrow diketo adipic acid \rightarrow succinic acid + formic acid \rightarrow
malic acid. The former reaction sequence demands an output of 1 millimol of carbon dioxide per mg. equivalent of acid formed and, as this amount is not produced, the upper schema is discarded. Toeniessen & Brinkman (49) showed that when liver was perfused with pyruvic acid this disappeared and succinic acid and traces of formic acid appeared but not in the theoretical proportions demanded by the lower schema. Diketo adipic acid was not detected nor is it known what the fate of diketo adipic acid in liver tissue is; so, even in liver tissue, one can hardly regard the Toeniessen-Brinkman schema as established. Diketo adipic acid has not been detected nor has its fate been investigated in plant tissues. Formic acid certainly cannot be detected even in traces in the tissues of malic-acid-accumulating plants. Nevertheless, this Toeniessen-Brinkman schema has been applied by Ruhland, Wetzel & Wolf as their explanation of formation of four-carbon acids in succulent plants.

They assume further that this conversion of pyruvic to malic acid is due to inhibition of carboxylase (which would otherwise convert the pyruvic acid to acetaldehyde) by the "abnormally large" concentration of aldehyde present in the leaves of succulents. Actually the aldehyde content of the tissues of succulents is remarkably small [Bennet-Clark (8), Gustafson (27), Kakesita (31)], usually about 0.01 to 0.001 per cent. Such concentrations have no inhibiting action on carboxylase [Wetzel (58)].

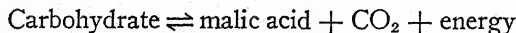
Certain hypotheses regarding the fate of the acid disappearing from tissues are equally unsupported by experimental evidence. Ruhland, Wetzel & Wolf consider that malic acid is converted into oxalacetic acid and, by loss of carbon dioxide to pyruvic acid, which, if they are correct, could either be converted back into malic acid or to acetaldehyde, the fate of which, it is suggested, is resynthesis to carbohydrate.

Acid disappears from leaves both when illuminated and also when kept in long-continued darkness. Thus, tissue placed in darkness at constant temperature at first accumulates acid; later (after about ten hours) this acid disappears. The acidity-time curve closely resembles the concentration-time curve of the reactant B in the consecutive reactions $A \rightarrow B \rightarrow C$ where the first velocity constant exceeds the second. Bennet-Clark (8) put forward the view that accumulation of acid was due to the relative activities of the enzyme systems involved in formation and loss of acid. The disappearance of acid in darkness is a peculiar process for it was shown that loss of acid was very high in comparison with carbon dioxide output. In a typical case, loss of 16 mg. of malic acid carbon (per hour per 100 gm. fresh weight) was associated with an output of 3.3 mg. of carbon dioxide carbon. It is therefore quite clear that the reaction, malic acid \rightarrow oxalacetic acid \rightarrow acetaldehyde $+ 2 \text{CO}_2$, does not take place. Loss of acidity, i.e., loss of carboxyl groups, cannot be due to evolution of carbon dioxide from these groups and neutralisation of acid is impossible in detached leaves; there remains the alternative that carboxyl groups are reduced to carbonyl and that the hydroxy-aldehydes so formed are polymerised to carbohydrate.

This view is supported by the observed values of the respiratory quotient (6), which during the phase of acid disappearance frequently exceed 2.0. Such high values should not be found if the reaction, malic acid \rightarrow acetaldehyde $+ 2 \text{CO}_2$, occurred, but are expected if the reaction, malic acid $- \text{O}_2 \rightarrow$ carbohydrate, occurs. The preparation of a complete balance sheet showing the fate of all the carbon and oxygen was unsuccessful owing to the difficulties of the carbohydrate analyses. The following schema was put forward as a description of the results with the emphasis that there is insufficient evidence as to the details of the several reactions:



The schema may be abbreviated to



This should conform to the Le Châtelier principle and the equilibrium should be displaced by rise in temperature in the direction in which

energy is absorbed. Disappearance of acid when the temperature is raised is in fact observed and the converse also (8).

Tobacco (Nicotiana).—The acid metabolism of tobacco is briefly noted by Schwartze who considers, on the basis of isolated pairs of analyses, that diurnal periodicity similar to that of the succulents occurs.

There has been a notable series of contributions by Vickery and coworkers (51, 52, 53). Many of these papers are concerned with analytical methods and qualitative recognition of various cell constituents and these form a basis invaluable to workers on *Nicotiana* as well as on other plant tissues. A most valuable developmental study (54) has been completed. The change in quantity, as the plants grew older, of dry weight, fresh weight, soluble and insoluble carbohydrate; malic, citric, oxalic, and unknown ether-soluble acid; pre-existent ammonia, ammonia produced by hydrolysis at pH 7, by 1N, and by 6N H_2SO_4 ; total N; nitrate N; etc., were determined. Broadly, one may say that the growth curves for all these constituents are parallel and sigmoid: growth is slow during the first forty days, rapid from the fortieth to the seventieth day and slow from then onwards. Thus, while the quantities of all the constituents, including the organic acids, increase enormously during the period of rapid growth, their concentrations remain roughly constant. The ratio of concentrations of malic: citric: oxalic: unknown ether-soluble acid is roughly 100:13:21:21 in the leaves, and 100:7:55:200 in the stems, and 100:25:25:230 in the young seed-pods. These ratios remain roughly constant through the whole life cycle and there is no accumulation of an acid which might be regarded as an end-product of a series of changes. The results suggest strikingly that the acids are maintained in equilibrium with each other and with other tissue constituents.

Rhubarb (Rheum).—Controversy of some importance surrounds the acid metabolism of rhubarb since its behaviour forms the chief line of evidence adduced by Ruhland & Wetzel (44, 45) in favour of their hypothesis that, in general, the plant acids are formed from amino acids (proteins) rather than from carbohydrate. The reasons for this view are broadly that (according to Ruhland & Wetzel) all the malic acid produced is formed during the period of sprouting of the rhizome, and that equimolecular amounts of ammonia and *l*-malic acid are formed in the young petioles simultaneously with a decrease in quantity of "rest-N," which is largely amino-acid nitrogen. They

state that no malic acid is formed in summer and that *dl*-malic acid is converted to oxalic and that in autumn excess *l*-malic acid is transported to the rhizome where it is inactivated during the phase of yellowing and final death of the leaves. Quantitative details in support of these statements, which appear in preliminary notes, are not given.

The results of Bennet-Clark & Woodruff (11) are completely opposed to the above view. During the phase of sprouting they found no increase in quantity of malic acid (or ammonia) in the plant though a very large increase occurred in the concentration of acid in the petioles and laminae, accompanied by a slight decrease in concentration in the rhizome and roots. It is believed that these concentration changes are due to transport of acid from the rhizome to the growing leaves. The initial high concentration of acid in the shoot decreased roughly logarithmically to a value which remained nearly constant throughout the summer. The period of most rapid growth occurred during this phase of constant acidity. Although the concentration of acid (gm. per gm. of dry weight or fresh weight) remains constant throughout the summer the quantity must obviously be increasing since the weight of the plant increases some six or seven times. These authors believe that Ruhland & Wetzel's conclusions are due to a confusion of concentration (gm. of acid per gm. of plant) with quantity (gm. of acid per plant). There was a fourfold increase in acid (largely *l*-malic acid) between June and September without any corresponding increase in ammonia; the ratio ammonia/malic acid was less than 0.1 throughout the whole season.

Results of Culpepper & Caldwell (18) also indicate that the acidity of rhubarb remains fairly constant during the growing period in summer, so that obviously large gains in quantity of acid must be occurring. The behaviour is closely similar to that of *Nicotiana*, cited above, and the evidence does not justify association of the organic acids with nitrogen rather than carbohydrate metabolism.

The death of the leaves in autumn is associated with a large increase in the quantity of acid in the underground organs. This accumulating acid is optically inactive and is, according to Ruhland & Wetzel, *i*-malic acid.

Naylor (37) has shown that rhubarb rhizome contains an "enzyme," active in alcohol preparations of the rhizome, which converts both *l*- and *d*-malic acids to the racemic *dl*-mixture, which was isolated and recognised as its hydrazide. *d*-Tartaric acid is not affected

by the enzyme; nor is fumaric, so the hypothesis that the enzyme is a mixture of *d*- and *l*-fumarases is incorrect. The mechanism and biological significance of this racemisation are quite unknown.

Tomato (Lycopersicum).—Clark (16) has investigated the influence of nitrogen nutrition on various features of the development of tomato, including its acid production. These results are summarised in Table I.

TABLE I
ACIDS AND AMIDES IN TOMATO STEMS* AND LEAVES†

	(mg. eq. per 100 gm. dry weight)					
	Oxalic Acid	Citric Acid	Malic Acid	Unknown Acid	Aspara- gine	Gluta- mine
NO ₃ ⁻	47(68)	28(11)	36(39)	42(29)	4(2)	3(3)
Conc. NH ₄ ⁻ ...	3(10)	1(1)	2(5)	65(50)	20(23)	20(81)
Weak NH ₄ ⁻ ...	(16)	(1)	(8)	(48)	(19)	(34)

* Figures in parentheses.

† Figures not in parentheses.

The plants were grown in cultures supplied with necessary salts, but with nitrogen as either nitrate or ammonium. It will be seen that plants cultured on ammonium salts produce almost no oxalic, malic, or citric acids, but that their unknown ether-soluble acid, asparagine, and glutamine are much increased. The significance of this is hard to interpret: it suggests that ammonia traps acids of the malic-oxalic group, or some precursor of them, with resultant amide formation, but it is not clear why the same equilibrium concentration of these acids should not be established in both nitrate and ammonium cultures. One is provided here with a very important experimental method, since, if the phenomenon is widespread, it should be possible to obtain plants rich in or almost free from acids of the malic group.

Begonia.—In a preliminary note on *Begonia*, Ruhland & Wetzel (43) also show that the oxalate content of nitrate cultures exceeds that of ammonium cultures. They state further that nitrogen-poor plants have twice as much carbohydrate as nitrogen-rich plants and a correspondingly more active respiration, yet they contain less oxalic acid. It is argued, therefore, that oxalic acid arises from amino acids rather than carbohydrate. Since the average composition of amino acids and proteins is 4 carbon atoms per nitrogen atom, one would expect a molar ratio, [oxalic acid]/[NH₃], of 2 just as the

ratio, $[\text{malic acid}]/[\text{NH}_3]$, was expected to be unity in the case of *Rheum*. Ruhland & Wetzel do not give figures in support of this. Shibata (47, 48) gives figures of the oxalic acid and ammonium content of *B. evansiana*; the $[\text{oxalic acid}]/[\text{NH}_3]$ ratio is about 2 in the tubers and stems, but it is about 10 to 20 in the leaves, and for the whole plant must obviously exceed 2. Such results might be brought into line with the hypothesis that oxalic acid is formed from the carbon residues of deaminated amino acids if various qualifying hypotheses were also introduced but they can hardly be regarded as evidence in its favour. Kultzscher (32) has extended our knowledge of the composition of a considerable number of plants, and has shown that, in general, plants with highly acid cell sap have abnormally large ratios, $[\text{ammonia}]/[\text{rest-N}]$; rest nitrogen in this case is water-soluble nitrogen other than ammonia and amide nitrogen.

These high ammonium contents of plants having saps of low pH value are explained by Ruhland & Wetzel as due to the great activity of their protein metabolism, in particular of their deaminase systems, and the low pH as due to conversion of the carbon residues into oxalic acid. There is obviously an alternative explanation which the published evidence does not exclude: that inherent peculiarity of the respiratory carbohydrate metabolism results in abnormal accumulation of oxalic acid (just as malic acid accumulates in the Crassulaceae); the resultant low pH would of necessity "fix" ammonia as a salt and would tend to cause abnormally great accumulations of ammonium ions in the sap, though probably the concentration of free undissociated ammonia is much the same in plants with very acid or more normal cell saps.

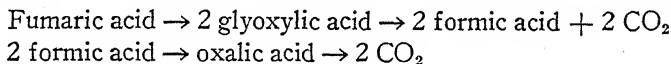
The mould fungi.—Various species produce organic acids. This aspect of plant acid metabolism was reviewed last year (30). A few new developments have taken place since then.

Bernhauer & Iglauer (12, 13) show that *Aspergillus niger* grown in neutral media "degenerate," i.e., lose their power of producing citric acid, but recover this power when grown on media made acid with mineral acids. They give an account of many of the conditions affecting citric acid yields and record yields up to 85 per cent of the sugar consumed. Clutterbuck (17) also reports yields up to 87 per cent of the sugar consumed. Butkewitsch & Gaevskaya (15) record 100 per cent yields of citric acid. The obtaining of yields over 71 per cent makes the widely accepted hypothesis that citric acid arises from aldehyde produced in alcoholic fermentation untenable. No new evi-

dence is yet forthcoming as to the mode of origin of citric acid in moulds.

The hypotheses of Franzen & Schmitt (26), Butkewitsch, and Virtanen (55) may be mentioned, but it must be remembered that they represent purely theoretical schemata which, in the latter two cases, are not even known to be possible *in vitro*. There is no evidence for the occurrence of any of them in the tissues of fungi or other plants.

Similar uncertainties surround the problem of the mode of origin of oxalic acid. It is very commonly stated that high yields of oxalic acid are obtained from acetic, glycollic, fumaric, and formic acids, among others, and in consequence various of these acids figure in hypothetical sequences of reactions, such as:



Actually oxalic acid is not obtained from any of these free acids but only when the fungus is grown on their salts. Under these conditions assimilation of the organic anion of the salts results in the production of free base which traps any oxalic acid produced by the mycelium. In fact the yield of oxalate is equivalent, as a rule, to the quantity of base available as a trap (1).

Allsopp (1) has investigated the mechanism of the normal formation of oxalic acid from sugar by an oxalic-acid-producing strain of *A. niger*. Oxalic acid production from sugar occurred in media made acid with mineral acids and which contained no base to trap the acid as oxalate.

Any genuine intermediate product in the conversion of glucose to oxalic acid should yield oxalic acid when fed to the fungus under similar conditions, namely, in acid solution containing no mineral bases. It is significant that among the substances which did *not* yield oxalic acid are the following: citric, malic, tartaric, succinic, fumaric, glycollic, acetic, and oxalacetic acids. These acids had no toxic effects for in all cases they were rapidly used up by the fungus; further, a dose of any of the above acids was found to have no effect on the rate of respiration of the fungus.

It seems, therefore, very improbable that any of the above acids are intermediates in the conversion of sugar to oxalic acid. It is well to emphasise that all reports in the literature of the formation of oxalic acid from four-carbon acids, etc., are based on the behaviour

of mycelia supplied with the salts of these acids; these salts certainly cannot be the intermediates in the conversion of pure glucose to oxalic acid since the necessary base is not present in the latter case. It should further be mentioned that a low pH of the medium is not responsible for the failure to produce oxalic from the four-carbon and two-carbon acids since glucose yields oxalic acid in media made acid with hydrochloric acid.

Allsopp accordingly suggests that oxalic acid is formed by splitting off the two terminal carbon atoms from such a compound as 2-ketogluconic acid, or analogous compounds which might be formed from other sugars. This is in agreement with the fact that gluconic acid, as well as glucose and a variety of other sugars, yield oxalic acid. A peculiar and as yet unexplained phenomenon noted by Allsopp is that oxalic acid formation from glucose is inhibited by lactic (and to a less extent by pyruvic) acid. There is also evidence that an equilibrium occurs which may be represented thus: carbohydrate \rightleftharpoons oxalic acid. Thus a mycelium supplied with oxalic acid at a concentration higher than the equilibrium concentration reduces this to the same equilibrium concentration which is attained when sugar only is supplied initially.

The older view that the organic acids produced by fungus mycelia are intermediates in the conversion of sugars to carbon dioxide has been criticised adversely. Bennet-Clark & La Touche (9, 10) showed that supply of citric, glycollic, and other acids to starving mycelia gave no extra carbon dioxide output though the acids were rapidly used up by the fungus. Supply of glucose on the other hand caused a marked rise in rate of respiration of the starving fungus. If these acids were intermediate products in conversion of sugar to carbon dioxide, they also would be expected to cause enhanced respiration when supplied to the culture. These results were confirmed and extended by Allsopp (1) who noted that the acids examined by him were utilised more rapidly by the fungus in the presence than in absence of glucose; this is in agreement with the view that the endothermic conversion of the acids to carbohydrate is coupled with the exothermic breakdown of glucose, and is in complete opposition to the view that the acids are intermediates in the conversion of sugar to carbon dioxide.

Such results indicate that the acid metabolism of the mould fungi is, at any rate superficially, very similar indeed to that of plants showing diurnal periodicity.

THE BIOLOGICAL RÔLE OF THE ORGANIC ACIDS IN PLANT TISSUES

Effective contributions to this aspect of the problem are a matter for the future, but many of the earlier suggestions are of considerable interest.

The first place should be given to the hypothesis of Liebig that the plant acids are intermediate products in the photosynthesis of sugars from carbon dioxide. This view has been restated in a variety of subsequent forms which differ in the precise formulation of the sequence of reactions. The primary stage is in all cases the photochemical conversion of carbon dioxide to oxalic acid. The possibility of occurrence of this photochemical reaction has been demonstrated *in vitro* by Baur (3). The demonstration that oxalic acid in the fungi, and malic and citric acid in plants showing periodicity, are convertible into carbohydrate makes a reconsideration of this unfashionable hypothesis desirable. In favour of the Liebig hypothesis one may mention that the energy change in the reaction $2\text{CO}_2 = \text{C}_2\text{O}_4$ is so small that less than one quantum per carbon dioxide molecule converted would be required even when red light is used. The large energy change involved in the more generally accepted formation of formaldehyde from carbon dioxide has been a source of difficulty which has evoked many hypotheses in attempts to explain away the fact that 4 to 5 quanta per molecule of carbon dioxide are required.

It is worth noting that the Liebig hypothesis was discarded in favour of the formaldehyde hypothesis of Baeyer as a result of the acceptance of arguments [Euler (19)], the validity of which now seems very doubtful. Euler contended that since the malic acid of the Crassulaceae decreases in quantity by day and increases in darkness it could not be an intermediate product in the photosynthesis of carbohydrate. Actually more recent work suggests that the malic acid is converted to carbohydrate and that the different concentrations found at different times of day are due in part to temperature differences and possibly also to different oxygen tensions in the tissues (8, 60).

The majority of earlier workers have regarded the acid production of plants (in particular that of the much investigated succulent plants) as a somewhat abnormal type of respiration in which sugar is oxidised "partially" to malic acid instead of to carbon dioxide. Many of these hypotheses are well reviewed by Evans (20). Certain

biological advantages were supposed to accrue from this: namely, that the products of respiration were not evolved and lost from the tissues, and that they were available for photosynthesis next day. Modern work on the respiration and acid metabolism of the mould fungi and of plants showing diurnal periodicity tends to substantiate this point of view. In both of these groups the amount of energy liberated by conversion of sugars to acids greatly exceeds that liberated in processes which culminate in evolution of carbon dioxide and one can hardly escape the conclusion that formation of organic acids from carbohydrates involves reactions of the greatest importance in transfer of energy of respiration to vital purposes. That part of this energy is apparently used up in promoting the endothermic resynthesis of carbohydrate from organic acid is a feature the significance of which is still quite unknown. There is no reason to suppose that in other groups of plants the organic acids do not play a similar rôle, but equally there is no evidence that they do so, largely because the striking fluctuations in acidity characteristic of the first named groups are not found in other plants.

Benecke (5) introduced the view that the function of the organic acids was to combine with the base made available when nitrate was assimilated in protein synthesis. He pointed out, what later work has confirmed, that acid production of nitrate-manured plants greatly exceeds that of ammonium-manured plants. Actually there must be a very marked exchange of nitrate ions with anions from the plant, presumably bicarbonate, since the number of equivalents of nitrate assimilated considerably exceeds the number of equivalents of organic acids produced by the plant in any cases which have been examined.

One cannot but regret, in view of the evident importance of the formation of plant acids as a source of respiratory energy, that so little and such unsuccessful investigation has as yet been carried out on their metabolism.

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THE BIOCHEMISTRY OF BACTERIA*

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GENERAL STUDIES ON OXIDATIONS AND REDUCTIONS

The current work of Warburg & Christian on dehydrogenation processes by the "pyridine"-lactoflavin complex (1) seems to apply, at least in some cases, to bacterial oxidations. The first example of a decreased respiration resulting from diminished oxygen tension at a rather high level is reported by Schlayer (2) for *Pneumococcus* Type I. In an attempt at answering the question whether this respiration must be considered as a physiologically important process for the organisms, Schlayer compares aërobic and anaërobic growth and finds the former much faster. This would be conclusive evidence if the anaërobic experiment (2 vol. per cent oxygen) was carried out in the presence of sugar, the necessary substrate for anaërobic metabolism. This, however, is not clearly stated.

A study by Wieland & Pistor (3) on oxidations by the catalase-negative *Acetobacter peroxydans* is also of importance. Contrary to expectation, hydrogen peroxide could not be detected. The reason for this appears to be that the organism can use hydrogen peroxide, when present in low concentration, as hydrogen acceptor. At concentrations above 0.001 *M*, hydrogen peroxide inhibits the oxidation, both of hydrogen and of ethanol; the inhibition is nearly complete at 0.006 *M*. Oxidation of hydrogen proceeds faster with hydrogen peroxide than with oxygen; in the latter case an inhibition soon becomes apparent, although the oxidation of ethanol does not seem to be influenced. In the presence both of hydrogen peroxide and oxygen, hydrogen is not oxidized at all. The authors try to explain this curious observation by assuming a particularly strong affinity of oxygen for the active enzyme surface which decreases the adsorption of hydrogen peroxide. It would seem that this assumption does not explain the fundamental difference in behavior between hydrogen and ethanol oxidation, nor account for the initially rapid oxidation of hydrogen in the presence of oxygen.

A definite relationship between metabolism and oxidation-reduction potential has been demonstrated in a number of cases [Kluyver &

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Hoogerheide (4, 5); Longworth & MacInnes (6); Korr (7)]. Thus the potential produced by organisms causing a typical alcoholic fermentation (yeasts, *Pseudom. Lindneri*) is independent of the organism, and reaches a value corresponding to $rH \pm 9$, while that caused by numerous strains of homofermentative lactic acid bacteria corresponds to rH 5.0 to 5.8. Of great consequence is the observation that the characteristic potential is not always established at the electrode unless redox indicators to which the cell is permeable are added. In their discussion of this behavior Kluver & Hoogerheide remark that not all redox systems which function in metabolism can be expected to be partly excreted. Because the electrode potential is governed by those systems which are present outside the cells, this may, therefore, give rise to a potential which is not immediately related to the entire set of functional redox systems. Hence a "universal indicator mixture," covering a wide potential range, has been developed, and by adding this to the medium consistent results have been obtained. The potentials determined by Longworth & MacInnes in anaerobic cultures of *Lactobacillus acidophilus* without added indicators agree with the data of the first-mentioned authors for cultures with the indicator mixture.

The effects of various factors on the oxidation-reduction potential of luminous bacteria have been studied by Korr (7), and bear out the idea that the potential is determined by kinetic factors (reaction rates of the positive and negative dehydrogenase-substrate systems).

Mention should also be made of the study by Cozic (8) on the potential produced by metabolizing acetic acid bacteria (rH 21 to 23) and of that by Wood, Wood & Baldwin (9) on the growth of *Bac. megatherium* as dependent upon the redox potential of the medium.

An apparent discrepancy emerges from an investigation on the effect of potential and oxygen content of the medium upon the growth of a butanol clostridium [Knaysi & Dutky (10)] which develops only if the potential drops below 300 mV. at pH 6.8, whereas it will grow in the same medium if its potential, in the absence of oxygen, is kept slightly below 335 mV. by the addition of potassium ferricyanide. No attention was paid, however, to the fact that the pH drops considerably following addition of the ferricyanide¹ so that the discrepancy is only apparent.

¹ M. Darsie measured: pH without potassium ferricyanide, 6.8; with 0.05M $K_3Fe(CN)_6$, 6.2. Calculations show that rH for limiting growth conditions in both cases is 23.6.

Of considerable theoretical and practical significance is a study on the oxidative metabolism of *Prototheca* [Barker (11)], fully corroborated by Giesberger's extensive investigation of the genus *Spirillum* (12). The results show that a single organic substrate, even in a non-nutritive medium (aqueous cell suspension), is at first only partly converted into the normal excreted oxidation products, and that a proportion varying from 50 to over 80 per cent, depending upon the nature of the substrate, is simultaneously converted into cell materials; probably the latter are reserve materials primarily. This means that, even when using washed cell suspensions and the manometric technique, one is not studying catabolic reactions only, but that also in these cases anabolism plays a quantitatively very important rôle. The results obtained in these studies suggest that the anabolic reactions initially lead to the formation of carbohydrate, although the *Spirillum* species also synthesize volutin during the oxidation of fatty-, hydroxy-, and dicarboxylic acids. The mechanism of the conversions is by no means clear; whereas, e.g., acetic acid is converted by *Prototheca* and the various *Spirillum* species according to the equation



the readily conceivable intermediate oxidation products, glycollic, glyoxylic, and succinic acids are not attacked at all by *Prototheca*.

The connection between anabolism and catabolism, here established, together with the determined ratio of the numbers of substrate molecules oxidized, oxygen used, and carbon dioxide produced, will prove of great value in establishing the mechanism of anabolic processes, and seems to rank equally in importance with kinetic studies in photosynthesis.

Additional papers, pertaining to this same problem, but less completely worked out (13, 14, 15), will only be mentioned here as general corroborative evidence. Of special interest is the study by Webster & Bernheim (14) on the oxidation of amino acids by *Pseudomonas aeruginosa* from which it appears that the oxidation of these compounds also gives rise to quantitatively very important anabolic reactions.

NITROGEN FIXATION

Winogradsky (16) has summarized his previous work on nitrogen fixation by *Azotobacter* and discussed the experimental evidence for the idea that ammonia is the first detectable product. Of great im-

portance for this problem is the publication of Burk & Horner (17) in which Winogradsky's claim is carefully examined and the experimental details are given which strongly favor their previous opinion.² They conclude: "The ammonia observed has been liberated *after*, not *before*, a synthesis into cell nitrogen. The occurrence of any ammonia as an essential intermediate product in the fixation of nitrogen, *although possible, still remains to be demonstrated.*"

In complete agreement with their findings are the results obtained by Roberg (18). This author has also reported the entirely negative results furnished by a study of the enzymatic nitrogen fixation (19).

Various papers deal with the effect of environmental factors (metals, colloids, etc.) on *Azotobacter* (20, 21, 22). The stimulating effect of small quantities of agar, and apparently not due to traces of metals [Rippel & Lehmann (22); Virtanen (23)], deserves further study because it remains entirely unexplained. During the past few years Stapp & Bortels (24) have noticed a striking dependence of *Azotobacter* development on the weather conditions; it may be mentioned here that this seems in line with recent observations on the spreading of epidemics.

The problem of nitrogen fixation by leguminous plants and their root-nodule bacteria still remains where it was in 1893 [cf. Fred *et al.* (25), p. 81]. Previous claims of nitrogen fixation by the plants themselves under special conditions have been clearly discredited by Smyth & Wilson (26, 27) who have shown that the previously used method for nitrogen determination (Kjeldahl) does not give reliable results.

A careful investigation of the nitrogen-fixing ability of the root-nodule bacteria by Winogradsky & Winogradsky (28) has again proved that these bacteria do not behave as truly nitrogen-fixing organisms. The observed increase in nitrogen of cultures grown in the absence of combined nitrogen as well as in the presence of organic extracts are too small, though rather consistent, to justify any positive claims. The authors conclude that one gains the impression that *Rhizobium* might possess the physiological characters required for developing at the expense of molecular nitrogen, but that this faculty cannot manifest itself in the cultures on account of the lack of necessary, activating principles in the media, which are provided by the plant; hence the experiments with nodules, entire or crushed. From the fact that ammonia is so readily produced by these organs the con-

² Cf. *Ann. Rev. Biochem.*, 5, 548 (1936).

clusion is drawn that, in accord with the views developed for nitrogen fixation by *Azotobacter*, the source of this ammonia is most probably the atmospheric nitrogen. But this hypothesis implies an increase in fixed nitrogen in the system nodules-air. Now, the present study does not offer any proof for this most important consequence. The latest attempt at answering the question whether nodules are capable of fixing atmospheric nitrogen [Galestin (29)] has been decisively negative. Without an equally conclusive proof to the contrary it seems, therefore, reasonable to consider the ammonia formation by the nodules as due to the decomposition of nitrogenous constituents rather than as a direct product of nitrogen fixation.

SPECIAL OXIDATION PROCESSES

The biochemical activities of the acetic acid bacteria have been reviewed by Butlin (30) who has also shown the influence of environmental factors on glucose oxidation by *Acetob. suboxydans* (31). It is somewhat difficult to reconcile his results with those previously obtained. Thus it is stated that the organisms, if grown in the absence of calcium carbonate, produce gluconic acid exclusively, and that they have no action on gluconate at pH 5.5. If grown in the presence of calcium carbonate a decomposition of glucose and gluconate was effected with the production of carbon dioxide in an amount almost equivalent to that of oxygen used, which would indicate a nearly complete oxidation of the substrate. Butlin has tried to correlate these differences in metabolism with differences in phosphatase activity, and reports a somewhat higher phosphatase action in cultures grown in the presence of calcium carbonate. It must be remembered that *Acetob. suboxydans* was isolated as a species characterized by the extensive production of 5-ketogluconic acid from glucose in the presence of calcium carbonate. Apart from the possibility that the results of the manometric measurements may not allow of an unambiguous interpretation (cf. 11, 12), it is conceivable that the strain of *Acetob. suboxydans* used had undergone considerable changes since its isolation; the reviewer has observed such changes before.

Hermann & Neuschul (32) have reported the oxidation of mannose to mannonic acid by acetic acid bacteria with yields of 60 to 70 per cent, and Takahashi & Asai (33) that of galactose to galactonic and comenic acids; the latter product was formed in small amounts (0.6 gm. comenic acid per 100 gm. galactose). Of interest are the extremely high concentrations of substrate which can be effectively

oxidized by these bacteria; concentrations of 40 per cent glucose with about 50 per cent theoretical yield of gluconic acid (34), and of 35 per cent sorbitol with 80 per cent yield of sorbose (35) have been used.

The utilization of surplus apples for the manufacture of calcium gluconate with the acid of acetic acid bacteria has been investigated (36); the observed yields are rather poor because a large proportion of the sugar is present as fructose.

Staphylococcus and *Propionibacterium* species oxidize lactic acid to pyruvic acid [Sevag & Neuenschwander-Lemmer (37); Erb, Wood & Werkman (38)]. The former organisms seemed to exert no further action on pyruvic and acetic acids in culture flasks, whereas similar experiments in Warburg vessels led to quite different results; from the quantities of oxygen used and carbon dioxide produced one might conclude that a complete oxidation of lactic acid, glucose, and glycerol took place. Since, however, the amount of substrate oxidized has not been determined, it is impossible to evaluate these results. The *Propionibacterium* species (38) decompose pyruvic acid to acetic acid and carbon dioxide.

Oxidation of amino acids.—The study by Webster & Bernheim on the oxidation of various amino acids by *Pseudom. aeruginosa* with the simultaneous synthesis of cell material (14) has already been referred to. Alanine oxidation under anaërobic conditions in the presence of nitrate by non-specified soil bacteria, probably *Pseudomonas* species, leads chiefly to ammonia and pyruvic acid, the nitrate being reduced to nitrite [Aubel & Egami (39, 40, 41)]. The occurrence of small amounts of carbon dioxide and acetic acid shows that the pyruvate can be further oxidized. A comparison has been made between the effects of various poisons and narcotics on the oxidation of alanine and lactic acid in the presence of methylene blue and nitrate under anaërobic conditions. For the dehydrogenation of alanine the nitrate cannot be replaced by methylene blue, in contrast to that which holds for lactate oxidation. A striking difference in the behavior of oxidations with nitrate and with methylene blue as acceptors is that only the former are inhibited by cyanide, octyl alcohol, and toluene.

Decomposition of cystine in an otherwise purely mineral medium by an organism obtained from enrichment cultures in the same medium leads to ammonia, free sulfur, and carbon dioxide, in a ratio of about 1:1:3, i.e., the theoretical one for complete oxidation [Barber & Burrows (42)]. No indications were obtained of the formation of sulfhydryl groups or hydrogen sulfide as precursors of the sulfur.

The decomposition occurs rather slowly, 0.05 per cent of substrate being oxidized in seven days.

Majima's studies on the breakdown of tryptophane derivatives (43, 44) offer nothing new. Bauguess (45) showed that diphtheria bacteria can utilize *d*- and *l*-tryptophane equally well, contrary to what holds for *B. coli* and others.³ *B. coli* causes a complete dephosphorylation and deamination of adenosinetriphosphoric acid, and of muscle and yeast adenylic acid; adenine and adenosine are deaminated [Lutwak-Mann (46)]. These decompositions occur aërobically and anaërobically, also in the presence of toluene. In some cases the previous history of the organism is, however, not without effect: *B. coli* grown on tryptic broth with 0.5 per cent glucose failed to attack adenosine triphosphate. The end product of all these decompositions is hypoxanthine.

The same strain of *B. coli* that produces tyramine and histamine respectively from *l*-tyrosine and histidine, can, according to Hirai (47), also produce putrescine from *d*-arginine. Although this would not be improbable, the reported yield of pure putrescine (7.5 gm. from 10 gm. arginine) is so much in excess of the maximum theoretical yield (6.0 gm.) that grave doubt is cast on the purity of the isolated product.

A decomposition of chitin by crude cultures of halophilic bacteria has been observed by Stuart (48) who did not succeed in isolating a pure culture with the same property.

Virtanen & Laine (49) have made some studies on the breakdown of proteins by root-nodule bacteria. A very slow increase in soluble and amino nitrogen is the result of the bacterial activities on milk, caseinogen, gelatin, and cell proteins in the presence of rather large amounts of sugar.

Bacterial oxidation of hydrocarbons with the production of (unidentified) acids has been found to occur both for petroleum [Tausson & Shapiro (50)] and rubber [Spence & van Niel (51)].

A study of the various oxidation processes taking place in sewage purification by means of activated sludge with the aid of the manometric technique has been started [Wooldridge & Standfast (52, 53, 54, 55)]. The low respiratory quotient (0.22 to 0.56) which, according to the authors, would indicate incomplete oxidation should be mentioned. Because carbon-dioxide determinations do not include

³ Cf. *Ann. Rev. Biochem.*, 5, 547 (1936).

estimation of dissolved carbon dioxide these values may, however, be meaningless. The rate of oxygen absorption for a mixture of sludge and sewage is greatly dependent on the condition of the former; sludge previously aerated is much more active following the addition of sewage than the same material saturated with sewage just before. Washing of the latter sort gives a product which takes up oxygen at a rate independent of the amount of sewage added. These experiments confirm the long held view that in activated sludge treatment adsorption occurs first and is followed by a biochemical, chiefly bacterial, oxidation of the organic material. By careful treatment of a bacterial suspension or crude sewage with formaldehyde or ultraviolet light one may obtain a material which, although not showing colony development on solid media, is still capable of oxidizing the substrate, a nice demonstration of the activity of enzymes. The effect of the protozoa, always found in activated sludge, is discussed, and an experiment is reported which would throw doubt on the previously expressed idea [Butterfield & Purdy (56)] that the protozoa render the sludge more active by continually keeping the bacterial population just below the saturation point, and thus insuring continued growth of the latter. Unfortunately, Wooldridge & Standfast used a culture of *Polytoma uvella*, which is not a bacterial feeder, for this experiment, so that the result is valueless in this connection.

BACTERIAL LUMINESCENCE

An addition to Korr's study, mentioned before (7), is the work by Taylor (57) on the effect of narcotics on the respiration and bioluminescence of luminous bacteria. From the results one may conclude that the inhibition of these two processes cannot be attributed to a simple adsorption process.

Purified luciferin [Anderson (58)] has been used in an investigation on the oxidation of the light-producing substance [Anderson (59)]. Evidence is presented for the hypothesis that luciferin can be reversibly oxidized by some oxidizing agents, but that these oxidations are different from the luminescent one; the end products of luciferin oxidation by oxygen in the presence of luciferase appear to be quite specific. "It means that until something more definite can be said about the light-producing reaction, no reliable basis is present on which to calculate the energy available for light emission." Some speculations are made on the chemical nature of luciferin, partly based upon a preliminary value for the apparent oxidation-reduction

potential of the luciferin system. Korr (60) has also demonstrated the oxidation of luciferin, which is at least partly reversible, and the unusually positive oxidation-reduction potential.

It has long been known that luminescence is dependent upon oxidative processes. This interrelationship has been somewhat clarified by Johnson (61) in a study of the respiratory activity of two luminous bacteria in which an inhibitory effect on the rate of oxidation of certain sugars was demonstrated due to the addition of non-oxidizable carbohydrates. The ideas of Quastel [cf., e.g., (62)] on the competitive adsorption of structurally related compounds by enzyme surfaces can readily be applied here. The dependence of luminescence upon respiratory reactions follows from the following experiments. The addition of oxidizable carbohydrate to a suspension of washed cells that had become dim through long aëration, restores luminescence. In addition, the inhibition of glucose oxidation by α -methyl glucose also inhibits the maintenance of luminescence, whereas, with the reversal of such inhibition, luminescence reappears.

All the above-mentioned data strongly suggest that luciferin can readily be regenerated from its biological oxidation product through the oxidation of a substrate, from which it would follow that the luciferin-oxyluciferin system might act as a redox system in the organisms. This is still more clearly brought out by the important contribution of van Schouwenburg & Eymers (63) on the effect of cyanide on both oxygen consumption and light intensity. It appears that oxygen consumption consists of two parts; one is completely inhibited by 0.001 *M* KCN, the other decreases slowly with increasing potassium cyanide concentration, and in proportion to light intensity. A study of the absolute quantity of oxygen used during the second part, and of the corresponding light emission in absolute units, led to the surprising result that per quantum of light emitted 500 molecules of oxygen are used. The authors conclude:

It seems remarkable that, whereas the percentage of O_2 consumed in the light-emitting process proved to be constant under different conditions and at different temperatures, the light intensity varies a great deal. It must be concluded, therefore, that the efficiency of the light-emitting process is dependent on various, as yet unknown, factors.

The last-mentioned investigators have also made a careful quantitative study of the spectral energy distribution of the light emitted by *Photobact. phosphoreum* (64). This appeared to be fully independent of external conditions (temperature, acidity and salt con-

tent of medium, age of culture), proving that the production of light can be ascribed to one chemical reaction which involves oxygen consumption. The spectrum was compared with that of two chemiluminescent reactions, the oxidation of 3-aminophthalhydrazide and of dimethyldiacridylum nitrate. At first sight there is no similarity between these spectra, but an analysis on the basis of the assumption that the energy distribution is due to symmetrical broadening of fundamental frequencies in the aqueous medium brings out the fact that the fundamental frequencies of bio- and of chemiluminescent reactions are limited and shared by many of them. The following table shows the results.

TABLE I
SPECTRAL DISTRIBUTION OF EMITTED LIGHT

Spectrum	Fundamental Frequencies in cm^{-1}					
<i>Cypridina</i>	21250	20200
<i>Lampyris</i>	18200	17600	16700
<i>Photuris pennsylvanica</i>	18200	17600
<i>Photinus consanguineus</i>	17600	16700
<i>Photinus pyralis</i>	17630	16200
<i>Photobact. luminosum</i>	20400	18300
3-Aminophthalhydrazide	21250	20300
Dimethyldiacridylum nitrate	20200	18350

This fine piece of deduction ("With due consideration of the preliminary character of these results, it is obvious that the fact that the energy frequency curves of bioluminescent processes occurring in so widely different groups of organisms have so much in common, cannot be incidental") strengthens the belief that a "more systematic study of the physics of chemiluminescent reactions proceeding in aqueous media will yield interesting results regarding the mechanism of biological light emission."

GENERAL STUDIES ON ANAEROBIC CARBOHYDRATE METABOLISM

The new schemes for intermediate anaerobic carbohydrate metabolism developed since 1933 [cf. Meyerhof & Kiessling (65)] have led to additional inquiry into the possible application of the same mechanism in bacterial fermentations. The focal compound which has replaced methyglyoxal, phosphoglyceric acid, has been isolated from

fermentations by lactic acid bacteria, propionic acid bacteria, and organisms of the coli-aerogenes group [Stone & Werkman (66, 67)]. Furthermore, the fermentation of pyruvic acid by *Lactobac. (Betalact.) lycopersici* with the production of 1 mol of lactic acid, acetic acid, and carbon dioxide each, from 2 mols of pyruvic acid [Nelson & Werkman (68)] is easily explained on this basis, as is also the result of studies by the same authors on the fermentation of glucose alone and in the presence of added acetaldehyde or acetylmethylcarbinol (69, 70), which latter act as competing hydrogen acceptors. Whereas the applicability of the Embden-Meyerhof scheme is realized, it is not clearly expressed in the proposed schemes, in which pyruvic acid is derived from lactic acid by dehydrogenation instead of functioning as the precursor of lactic acid.

Also, the fermentation of pyruvic acid by bacteria of the coli group [Mickelson, Reynolds & Werkman (71)] may be taken as evidence that this compound is the precursor of lactic acid. Yet, the nature and ratio of the decomposition products show that a splitting of the keto acid is quantitatively far more important than its coupled reduction. Thus, *Bact. coli* produced 15.6 millimols of lactic acid per 65.5 millimols, and *Bact. (Citrob.) Freundii* only 6 millimols per 48.2 millimols of pyruvic acid fermented. The rest of the fermented substrate is accounted for as acetic and formic acids, carbon dioxide, and hydrogen. The difference between the number of molecules of carbon dioxide and hydrogen is practically the same as the number of molecules of lactic acid formed, so that the following reactions are postulated:

1. $\text{CH}_3 \cdot \text{CO} \cdot \text{COOH} + \text{H}_2\text{O} \rightarrow \text{CH}_3 \cdot \text{COOH} + \text{HCOOH}$
2. $\text{HCOOH} \longrightarrow \text{CO}_2 + \text{H}_2$
3. $\text{CH}_3 \cdot \text{CO} \cdot \text{COOH} + \text{H}_2 \rightarrow \text{CH}_3 \cdot \text{CHOH} \cdot \text{COOH}$

Aerob. aerogenes produced from 50.6 millimols of pyruvic acid 28.8 millimols of acetic acid, 9.4 of 2,3-butylene glycol, 48.5 of carbon dioxide and 24.85 of hydrogen; no lactic acid was formed. The glycol is supposed to arise by reduction of acetic acid via acetylmethylcarbinol. It may be remarked that no evidence exists for the reduction of acetic acid to acetylmethylcarbinol; all the existing evidence points clearly to its formation through a condensation of acetaldehyde, and the reduction of acetic acid to aldehyde by *Aerob. aerogenes* has not yet been demonstrated.

REDUCTION OF CARBON DIOXIDE BY BACTERIA

To the conflicting views concerning the metabolism of the purple sulfur bacteria⁴ has been added the explanation of the discoloration of reduced dyes under the influence of illuminated cultures of *Chromatium spec.* as due to oxygen production [Czurda (72)]. If this interpretation were correct it would necessitate a complete revision of the theory of bacterial photosyntheses.⁵ This, however, does not seem necessary. It could be shown [van Niel (73)] that discoloration of the reduced dyes depends upon direct contact with the organisms, so that Czurda's interpretation appears untenable. Also, the situation with respect to the reduction of carbon dioxide by *Thiorhodaceae* in the presence of organic substrates and of hydrogen has been cleared up (73). Gaffron's results⁴ have been substantiated but his explanation was rejected on the following basis:

- a) Hydrogen sulfide production in the dark takes place only when the organisms are stuffed with sulfur. This process appears as a quantitatively insignificant side reaction ("phytochemical reduction").
- (b) Direct assimilation of carbon dioxide and organic substrates or hydrogen takes place if the medium is kept sufficiently alkaline.
- (c) Manometric readings of carbon dioxide absorption are greatly influenced by the regular production of acid substances which tend to obscure the uptake of carbon dioxide.

Quantitative analyses of purple bacteria have proved that the cellular constituents are distinctly more reduced than carbohydrate, the percentage of carbon and hydrogen corresponding remarkably closely to Gaffron's product $(C_4H_6O_2)_n$.

In a recent note, French (74) has published results of studies on the quantum efficiency of purple bacteria photosynthesis. The reaction studied was the photochemical carbon dioxide reduction with hydrogen by *Streptococcus varians*. Although the reaction is slightly exothermic, it does not take place in the dark, but apparently requires about four quanta of light for the reduction of one molecule of carbon dioxide.⁶

Heretofore, carbon dioxide reduction has been considered as the outstanding and exclusive characteristic of photosynthetic and chemo-

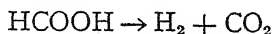
⁴ Cf. *Ann. Rev. Biochem.*, **4**, 610 (1935); **5**, 541 (1936).

⁵ *Ann. Rev. Biochem.*, **2**, 488 (1933); **3**, 529 (1934).

⁶ Cf. *Ann. Rev. Biochem.*, **4**, 610 (1935); **5**, 541 (1936).

autotrophic organisms. This year's literature shows conclusively that this view can no longer be upheld.

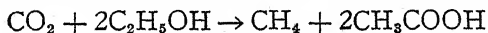
There is the fine contribution by Woods (75) on the reduction of carbon dioxide to formic acid by molecular hydrogen under the influence of *Bact. coli*, proving that the reaction



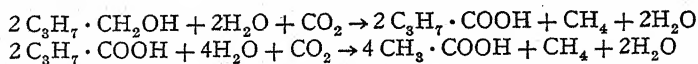
is a truly reversible process, and that the synthesis of formic acid—just as its decomposition—can be brought about only with organisms grown in the presence of formate.

In addition, Wieringa (76) has communicated the isolation of an anaerobic spore-forming organism which produces almost quantitatively acetic acid out of carbon dioxide and hydrogen. Because this organism cannot decompose acetate with the formation of methane and carbon dioxide the earlier reports on methane fermentation from carbon dioxide and hydrogen⁷ may have to be explained as the result of the co-operation of at least two bacterial species.

Nevertheless, the methane fermentation itself is a typical process of carbon dioxide reduction. This has been established beyond a doubt in an excellent study by Barker (77, 78) who found that one species of methane-producing bacteria can carry out the reaction:



Hence, the dehydrogenation of ethanol to acetic acid takes place with carbon dioxide as the only final acceptor. If butanol is used as a substrate instead of ethanol then the first dehydrogenation product is butyric acid, and its production is accompanied by the theoretical amount of reduced carbon dioxide, i.e., methane. In later stages the butyric acid is converted into acetic acid, and again the only corresponding reduction product is methane. The two successive stages of the fermentation of butanol are represented by the following equations:

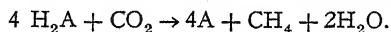


Barker concludes:

The experimental results provide adequate proof that methane actually arises by a reduction of CO_2 in at least the special cases of the fermentation of ethyl and butyl alcohols, and probably also of butyric acid. It seems only logical to

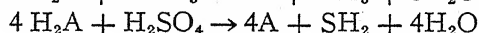
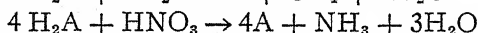
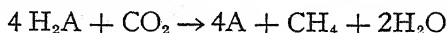
⁷ Cf. *Ann. Rev. Biochem.*, 2, 492 (1933).

generalize this result to include all fermentations of this type. The general equation for the methane fermentation would, therefore, be



In this equation H_2A represents any compound, organic or inorganic, which can be activated by the methane bacteria so that it can act as a hydrogen donor for the reduction of CO_2 .

The methane fermentation as a process of carbon dioxide reduction thus appears closely analogous to the well-known process of nitrate and sulfate reduction:



Perhaps the greatest significance of these studies is to be found in their ultimate connection with other processes of carbon dioxide reduction.

Wood & Werkman (79) claim that carbon dioxide is reduced during the fermentation of glycerol by propionic acid bacteria. The published results cannot, however, be considered conclusive, although the data do seem to favor their claim.

SPECIAL FERMENTATIONS

Lactic acid fermentation.—Production of acetylmethylcarbinol (and diacetyl) under the influence of lactic acid bacteria [Matuszewski *et al.* (80)] may take place directly from sugar because the addition of citrate greatly decreases the amount of carbinol formed.⁸ The reduction of the chief component of butter flavor and its precursor to 2,3-butyleneglycol (69, 70) has been further studied by Hammer *et al.* (81, 82). Obviously this reduction depends upon the dehydrogenation of other compounds, so that it is not surprising to find that conditions most favorable for the normal metabolic activities of the organisms (such as neutralization of the medium) cause a greater reduction of the carbinol than is observed under unfavorable conditions (high acidity, low temperature) or following the addition of competitive hydrogen acceptors, hydrogen peroxide, fumarate).

Comparative studies of considerable extent on lactic acid bacteria in relation to carbinol production and their fitness for use as starters, have been carried out by van Beynum & Pette (83), while Smit

⁸ Cf. *Ann. Rev. Biochem.*, 5, 550 (1936).

(84, 85) has published a simple method for differentiating the common bacteria in starters. Hammer (86) has studied the possible production of higher homologues of acetylmethylcarbinol as a result of the addition of various aldehydes to cultures of *Streptococcus liquefaciens*. Although the yield of acetylmethylcarbinol was increased, no evidence for the formation of higher homologues was obtained.

The industrial manufacture of *d*-lactic acid by the use of strains of lactic acid bacteria which produce only the *d*-isomer seems feasible [Tatum & Peterson (87)]; up to 10 per cent sugar can be completely fermented in the presence of calcium carbonate. A curious racemization of optically active lactic acid is effected under the influence of *Clostridium* species [Tatum, Peterson & Fred (88)]. This racemization is not accompanied by a fermentation of the acid, in fact it can be brought about by heat-killed cells, but only in the presence of a non-heated culture filtrate which by itself is without action.

Propionic acid fermentation.—Of considerable interest is the demonstration that propionic acid bacteria produce succinic acid from glycerol [Wood & Werkman (79)] and from glucose (89). This, together with the reported reduction of carbon dioxide has led to modifications of previously proposed schemes of the mechanism of this fermentation. The most notable among these are the formation of succinic acid by synthesis, possibly by the condensation of 2 molecules of acetic acid, the postulation of succinic acid as a truly intermediate product which may give rise to propionic acid and carbon dioxide [see also Stone, Erb & Werkman (90)], and the reduction of carbon dioxide to organic compounds. It is as yet impossible to evaluate the study on fatty acid activation by the propionic acid bacteria [Stone, Wood & Werkman (91)], and the merits of the proposed mechanism will have to be tested by further investigations. On succinic acid formation see also below (98, 99, 100).

Chaix & Fromageot (92) have continued their study of the disappearance of the "threshold phenomenon"⁹ upon the addition of sulfur compounds; the effect seems to bear no relation to the oxidation-reduction potential of the medium (activity of methionine!) and not to depend upon growth of the bacteria.

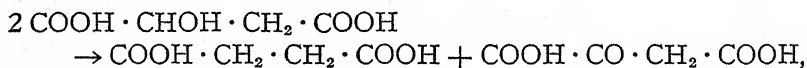
Fermentations of the coli-group.—The startling results reported a few years ago by Wieland and collaborators on the anaërobic decomposition of organic acids by yeast¹⁰ have now been shown to be due

⁹ Cf. *Ann. Rev. Biochem.*, 5, 553 (1936).

¹⁰ Cf., e.g., *Ann. Rev. Biochem.*, 3, 29 (1934).

to bacterial contaminations (93, 94). Renewed studies of these conversions have been made, using the contaminating bacteria for inoculating the media. The procedure followed for obtaining these cultures does not guarantee that pure cultures have been used, so that an interpretation of the results would be very difficult, if it were not for the fact that Barker simultaneously has published his investigation on the fermentation of some dibasic acids by pure cultures of *Aerob. aerogenes* (95). Especially because the latter has used a purely mineral medium with the addition of the acids only, thus avoiding complications arising from the use of yeast extract by Wieland and Sonderhoff, and because the enrichment cultures showed clearly that *Aerob. aerogenes* is the only organism which readily develops anaerobically at the expense of the organic acids, a discussion of these fermentations can be confined to Barker's study. This seems all the more justified on account of the qualitative agreement of the fermentation products. Of the acids tested (*d*-tartaric, *d*- and *l*-malic, fumaric, maleic, and succinic acids) only *d*-tartaric, *l*-malic, and fumaric acid are fermented. The products are the same as those obtained from carbohydrate fermentations with the exception of acetylmethylcarbinol and 2,3-butyleneglycol which were not encountered.

The yield of succinic acid in the fermentations of malic and fumaric acids (about 1 millimol per 2 millimols of fermented substrate) favors the concept that initially the decomposition proceeds according to the reaction:



followed by a decarboxylation of acetoacetic acid (fermentable!) and subsequent decomposition of the pyruvic acid into the common C_1 and C_2 compounds.

The production of succinic acid in sugar fermentations by *Bact. coli* is influenced by the amount of ammonium salts present [Michaelis (96, 97)], presumably because in the presence of high ammonia concentrations the formation of aspartic acid from succinic and fumaric acid is favored. The formation of the amino acid was not demonstrated, however, so that the interpretation remains doubtful. It must be observed that the difference in yield of succinic acid in some cases is so large that a considerable consecutive transformation of aspartic acid into cell proteins is out of the question, and that yields of lactic and acetic acids also vary considerably.

The interesting observations on anaërobic formation of succinic acid by animal tissue [Krebs (98) ; Weil-Malherbe (99, 100)] should be mentioned here because of their possible applicability to bacterial fermentations in which this product is formed.

Butanol fermentation.—Bernhauer *et al.* (101) have shown that in the presence of calcium carbonate the total quantity of butyric acid plus butanol is much greater than in its absence, while the reverse holds for total amount of acetic acid, ethanol, and acetone. This might mean that a change in the pH of the environment causes a shift in the type of decomposition, or that butyric acid is an intermediate, not only in butanol formation but also in the production of C_2 compounds. Hence the effect of two C_4 compounds was studied. Butyric aldehyde is, even in the presence of calcium carbonate, converted entirely into butanol; a dismutation does not seem to occur. Crotonic acid (with calcium carbonate) led to greatly increased yields of acetic acid; the same resulted from addition of pyruvate. Without calcium carbonate both substances caused a distinct increase in butyl products.

The results obtained by Hammer (cf. 86) furnish evidence that the argument advanced last year by Blanchard & MacDonald¹¹ against the hypothesis of butyric acid formation via an acetaldehyde condensation cannot be taken seriously.

A very unconvincing account is given of the fermentative abilities of a "new" butanol *Clostridium* (102).

Of importance in connection with the industrial application of the butanol fermentation is, in the first place, the study by Underkofler *et al.* (103) in which a procedure of "heat-shocking" is described which greatly improves the rate of fermentation, especially of xylose. The patent application (104) for the manufacture of tannic materials from sulfite waste liquors by fermentation with butanol-producing bacteria has the appearance of an attempt at obtaining a patent on the industrial application of this fermentation on secondary, unimportant features.

Anaërobic cellulose decomposition.—Two publications on this subject (105, 106) agree in that a vigorous fermentation by thermophilic organisms depends upon the presence of contaminating bacteria. Soeters has shown that the addition of an extract of *Bact. coli* makes possible the rapid development of a pure culture of the cellulose-fermenting bacterium. He also observed that the pure culture can ferment glucose only if this has been sterilized together with fecal extract.

¹¹ *Ann. Rev. Biochem.*, 5, 552 (1936).

This fermentation is still little understood; acetic acid, ethanol, carbon dioxide and hydrogen seem to be formed as the main products, with small amounts of butyric acid and butanol. Simakova (107) even reports a cellulose fermentation with propionic acid as the main product, accompanied by small amounts of valeric and formic acids.

Anaërobic breakdown of amino acids.—A continuation of Stickland's studies¹² by Woods (108) has revealed the existence of amino acids which can act as hydrogen donors (*l*-cysteine) or hydrogen acceptors (*d*-arginine and *d*-ornithine) but which undergo deamination also in the absence of special hydrogen acceptors or donors. Ornithine, in the presence of alanine as donor, is converted into δ -aminovaleric acid.

The generalizations to which the present information on these decompositions leads may be summarized as follows: Simple α -amino acids act only as hydrogen donors (except glycine). The presence of another substituent group leads to an abnormal behavior; such compounds can be broken down by *Clostr. sporogenes* in the absence of any other amino acid. Glycollic acid, β -alanine, and taurine cannot be used by *Clostr. sporogenes*, either as hydrogen donor or as acceptor.

NUTRIENT REQUIREMENTS OF BACTERIA

An important addition to the literature on this subject is Knight's comprehensive monograph (109). A concise statement of the problem is found in Lwoff's discussion (110). Great advances have been made, particularly in the nutrition of two bacterial groups, the lactic and propionic acid bacteria.

The necessity of carbon dioxide for the former organisms is proved by Longsworth & MacInnes (111). The investigations of Orla-Jensen *et al* (112 to 116) as also those by Eagles *et al.* (117, 118) show the exacting nature of the lactic acid bacteria by studies on acid production in still rather undefined media (milk and whey, treated with activated charcoal, etc.). By fractionation of extracts which exert a stimulating influence Eagles *et al.* obtained evidence that bios-I, -IIA, and -IIB are activators. The Danish investigators state that the lactic acid bacteria require not only bios (pantothenic acid) but also vitamin B₂ (lactoflavin). They demonstrate increasingly complex requirements for the *Streptococci*, *Streptobacteria*, and *Thermobacteria*. The first can use ammonia nitrogen as sole nitrogen

¹² *Ann. Rev. Biochem.*, 5, 554 (1936).

source, although addition of histidine, leucine, creatine, and yeast nucleic acid act favorably. The *Streptobacteria* require cysteine besides ammonium salts; also here, various additions (creatine, diketopiperazine, glutamic acid, sometimes histidine and lysine) may be useful. The *Thermobacteria* seem to need cysteine, tyrosine, lysine, histidine, arginine, glutamic acid, asparagine, and creatine.

The results of these studies are in some respects difficult to evaluate due to the fact that, firstly, not development but titrated acid formation was used as a criterion, and, secondly, because the media used in the experiments are still complex and undefined. The conclusions drawn by the investigators receive, however, considerable support from the investigations by Lwoff & Lwoff (119), Fromageot & Laroux (120, 121), and Tatum *et al.* (122, 123). Lwoff & Lwoff identified the "V"-factor for *Hemophilus* as cozymase or codehydrase, which must be considered a substantial confirmation of Pittman's idea (125) that the "V"-factor plays a part in the oxidative processes of *Hemophilus*.

The surprising ability of some lactic acid bacteria to build up their nitrogenous cell constituents from ammonia nitrogen is greatly corroborated by the clear demonstration that the closely related propionic acid bacteria can do so (121, 122, 124). Conclusive evidence here is the fact that the increase in nitrogen content of the bacteria is quantitatively accounted for by a decrease in the ammonia nitrogen of the medium. Generally, the propionic acid bacteria require in addition vitamin B₁. Some species seem to be capable of synthesizing this vitamin themselves (124).

Of general interest in connection with this problem is an investigation on the determination of bacterial numbers by plate counts (126). A synthetic medium yields parallel counts of abnormal variance, which could be ascribed to the irregular presence of organisms producing growth-promoting substances. Addition to the medium of extracts rich in such substances improved considerably the variability of the counts.

A growth-stimulating factor of unknown nature for growth of *Bact. coli* was described by Sahyun *et al.* (127). This is not a nutrient requirement because the organism can synthesize the activator.

BACTERIO-PHYTOCHEMISTRY

Substances giving a positive Feulgen test have been found in bacteria [Imšenecki (128)], proving the presence of characteristic

nuclear materials. The positive reaction is only observable with larger quantities of organisms, indicative of the existence of very small nuclei or of a diffuse distribution. This demonstration gains particular importance in view of the study by Schlesinger (129) in which a positive Feulgen reaction was obtained with concentrated phage substance. Schlesinger's claim: "So the phage substance seems to be chemically different from any constituent of the bacterial cell normally present in significant amount" thus loses its significance.

Eight new publications on the lipids of *Mycobact. tuberculosis* have been published [Anderson *et al.* (130 to 135); Bloch (136, 137)]. Phthioic acid ($C_{26}H_{52}O_2$), now characterized as a branched chain fatty acid, proved biologically active, and produced typical tubercular tissue upon injection; an optically active alcohol, phthiocerol ($C_{34}H_{67}(OH)_2OCH_3$), seems typical for human strains. The neutral esters of the acetone-soluble fats contain trehalose in place of glycerol. According to Bloch, Anderson's A3-phosphatide contains nitrogen only as an impurity (adsorbed ammonia) and can be described as the magnesium salt of a nitrogen-free phosphatide acid. This purified substance shows not only an increased biological activity; the appearance of non-specific lesions is also much diminished, whereas the specific effect becomes much more clearly perceptible.

Sifferd & Anderson (138) disclaim the occurrence of sterols in tubercle bacteria, and prove the production of ergosterol by *Azotobacter*.¹³

A specific polysaccharide giving precipitation with anti-B.C.G. sera has been obtained (139). The method, recently described for extraction of labile bacterial antigens by disruption of the cells at low temperatures [Czarnetzky (140)] may prove very fruitful in future studies on cell constituents.

Mention may still be made of studies on the carotenoids of purple bacteria¹⁴ [Schneider (141); Karrer & Solmssen (142, 143, 144)]; it must be remarked, however, that these studies have not been carried out with pure culture material, so that the claim (Karrer & Solmssen) that the composition of the carotenoid fraction varies greatly with the conditions under which the organisms were grown, does not imply that this holds for pure cultures.

The absorption spectra of the bacterial pigments, pyocyanin, pro-

¹³ Cf. *Ann. Rev. Biochem.*, 5, 555 (1936).

¹⁴ Cf. *Ann. Rev. Biochem.*, 6, p. 494.

digiosin, and violascein, have been measured by Erishmann & Noethling (145).

BACTERIAL BIOCHEMISTRY IN RELATION TO CLASSIFICATION

General studies have been published by Knight (109) and by Kluyver & van Niel (146). Special investigations on speciation in the group of lactic acid bacteria, partly in relation to their biochemical characteristics, include studies on *Streptob. plantarum* [Pederson (147); Hornbostel (148); Allen & Harrison (149)], *Bact. bifidum* and *Thermob. intestinale* [Orla-Jensen *et al.* (150)], and *Streptococcus lactis* [Stark & Sherman (151)].

Bact. pneumoniae has been characterized as a group of four types, one identical with *Aerob. aerogenes*, and all mutually distinguishable on the basis of the Voges-Proskauer test, development in citrate medium, and fermentation of glycerol [de Graaff (152)].

The genus *Spirillum* has been very adequately treated in Giesberger's monograph (12). Turfitt (153) found the chromogenic properties of *Ps. pyocyaneus* and *Ps. fluorescens* to be constant properties of the two species.

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IMMUNOCHEMISTRY*

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The following is a general survey of the literature during 1935 and 1936, with no claim to completeness. Among the books published in this period, mention may be made of a second edition of Topley & Wilson's *Principles of Bacteriology and Immunity* (1) which includes the revised material of Topley's *Outlines of Immunity*. A new periodical has appeared, *Revue d'Immunologie*, edited by Debré, Ramon, and Pasteur Vallery-Radot.

PROTEINS

Viruses.—The immunological method has again been applied for identification and differentiation of proteins. Of particular interest in this connection are studies on the nature of plant viruses. The high molecular crystalline protein [mol. wt. *ca.* 17,000,000 (2)] separated by Stanley (3; also cf. 4) from plants diseased with tobacco mosaic has been examined serologically, and the results are brought as additional evidence for its identity with the virus. This protein preparation from infected tobacco plants does not precipitate in antiserum produced with the juice of healthy plants, but engenders a precipitin active for itself, for the juice of diseased plants, and for the crystalline protein obtained from tomato plants infected with the same tobacco virus [cf. (5)]. Upon exposure to hydrogen peroxide, formaldehyde, nitrous acid or ultraviolet light, the protein loses its infectivity, but it is not denatured or changed significantly in its immunological properties, the material inactivated by ultraviolet light still giving rise to an antiserum which neutralizes the virus (6). [For further serological tests *vide* Chester (7).] The properties of another plant virus were studied by Spooner & Bawden (8). They found in the saps of several plants (tobacco, *N. glutinosa*, *Datura stramonium*, potato), when infected with the potato virus X, a common antigen which is not found in healthy tobacco or *Datura*, or in tobacco plants infected with several other viruses. The connection between the antigen and the infectivity of the potato virus has been indicated by Bawden (9).

* Received January 12, 1937.

Inactivation by treatment with nitrous acid [Bawden & Pirie (10)] or formaldehyde (9) is not accompanied by a change in the reactions with antiserum (the nitrite-inactivated virus indeed gives rise to neutralizing antibodies), but other inactivating procedures cause a loss of precipitability with antiviral serum.

Related to the question of the nature of viruses are investigations on bacteriophage [cf. (11)]. Apart from other evidence, it may be mentioned that Northrop (12) obtained from lysed staphylococci a protein preparation possessing the properties of, and not separable from bacteriophage.

The demonstration of two antigens in vaccinia virus which "behave *in vitro* as if components of a complex . . . antigen" [Craigie & Wishart (13)], and two antigens in psittacosis virus [Bedson (14)] suggests the existence of viruses not consisting of single chemical entities. This assumption would appear to be supported by the chemical studies of washed elementary bodies of vaccinia, in which Hughes, Parker & Rivers (15) found protein, carbohydrate, and fat.

Bacterial proteins.—Linton & Mitra have continued their studies on the cholera vibrios (16). Two types of vibrios were distinguished by the change in specific rotation of their globulins through the action of alkali, traced by Mitra (17) to differences in the racemization of certain amino acids. Taken in connection with three sorts of specific carbohydrates, this permits the establishment of six groups of vibrios. Seibert (18) has reported that a tuberculin preparation (SOTT), having a molecular weight of about 3800, was able to inhibit the precipitation of tubercle bacillus protein by its homologous antiserum. An extensive study of tuberculin and the antigens of tubercle bacilli in general has been presented by Sandor (19; cf. also 20). The antigenic structure of streptococci is being examined by Mudd *et al.* (21).

Toxins.—The neurotoxin of cobra venom has been studied by Micheel & Jung (22), by means of ultrafiltration, passage through cellophane, and precipitation. It appears to be akin to protein, although with a molecular weight estimated as low as 2500 to 4000; the older report by Faust on the saponin nature of snake venoms could not be confirmed. The substance apparently contains a reversible oxidation-reduction system. With diphtheria toxin, Eaton (23) effected considerable purification by precipitation with metal salts [see also (24)]. The purified material consists mainly of a protein having no cysteine sulfur and very little or no tryptophane. The method was later adapted to the purification of tetanus toxin.

Animal proteins.—Roepke & Bushnell (25) have confirmed serologically the occurrence in the serum of the laying hen of a phosphoprotein similar to or identical with vitellin in the egg yolk and its absence (or presence in small amounts) in the serum of the male. The value of precipitin tests in detecting small contaminations is shown by Goldsworthy & Rudd (26); from their experiments as much as 2 per cent of the protein in solutions of thrice crystallized horse-serum albumin may be globulin. Evidence for the existence in serum of two serologically different globulins is given by Harris & Eagle (27), the specificity according to their results being independent of lipids. Johnson & Bradley (28) showed that precipitins to globins react species-specifically with hemoglobins also [cf. Giovanardi (29)]. Immune sera to silk are described by Fell (30).

Altered proteins.—Several points inviting further examination have been brought out in investigations on modified proteins. Blumenthal (31) found that the precipitability of serum albumin is irreversibly decreased after treatment with thioglycolic acid to effect reduction of the disulfide groups, in contrast to ovalbumin in which the cyanide-nitroprusside test does not reveal "normally functioning sulfhydryl groups." As an explanation of the differences the author suggests either participation of the -SS- and -SH groups in the reaction, or a splitting of the antigen molecule by opening the -SS- linkage.

Experiments by Zoet (32), which appear to show that upon heating mixtures of horse and pig serum to 120° C. a new specific property is developed, would require confirmation.

The effect of formaldehyde upon serum proteins was reinvestigated by Horsfall (33). Due perhaps to a different manner of preparing the antigens from that used in earlier experiments where no pronounced change in species specificity was observed, antiserum to formalized rabbit serum precipitated formalized sera of various mammals, although to a lesser degree than the homologous antigen. This demonstrates again that almost any change in a native protein diminishes the original specificity (34).

The finding that di-iodotyrosine inhibits the precipitation of iodized protein by antiserum (Wormall) has been extended by Snapper & Grünbaum (35), who showed, by testing a variety of iodized aromatic compounds, that the essential structure is aromatic hydroxyl with iodine in the adjacent positions. Neither di-iodotyrosine nor thyroxine inhibited the reaction of thyreoglobulin with its immune serum (36).

CARBOHYDRATES, LIPIDS (HAPTENS)

Bacterial polysaccharides.—Definite progress has been made in the isolation and chemical characterization of serologically active carbohydrates.¹ In a thorough investigation Morgan (38) has isolated the specific polysaccharide of *B. dysenteriae* Shiga as an apparently uniform substance. It contains 1.6 per cent of nitrogen, in the form of acetylated amino groups, and on acid hydrolysis yields 97 per cent of reducing sugar; $[\alpha]_D = +98^\circ$. The evidence indicates that the complex polysaccharide is built up of basic structural units—repeated six times in the molecule—consisting of four hexose molecules and one N-acetylaminohexose. From iodine reduction it is inferred that the polysaccharide contains one free aldehyde group. The precipitability with antiserum is lost during the course of acid hydrolysis when only one-third of the reducing sugars is liberated, but at this point, as shown by inhibition tests, the products of hydrolysis still react with anti-Shiga serum. Another specific carbohydrate, that of *Pneumococcus* Type VIII, has been prepared by Brown (39) and Goebel (40). According to the work of Goebel, it is built up from glucose and aldobionic acid in the proportion of 5:2; the identification of its aldobionic acid with that from Type III polysaccharide may explain the cross reactions observed between the two specific carbohydrates. New information as to the constitution of the acetyl polysaccharide of *Pneumococcus* Type I is provided by Heidelberger, Kendall & Scherp (61). The basic unit in the molecule appears to be a trisaccharide “containing two molecules of uronic acid, possibly both galacturonic acid, and two atoms of nitrogen”; half of the nitrogen is free amino nitrogen. The presence of galacturonic acid in the molecule has now been definitely established by the isolation of its methyl glycoside.

With *Meningococcus* Type I, the specific substance is reported by Scherp & Rake (41) to be a sodium salt of a polysaccharide acid containing firmly bound phosphoric acid (nitrogen, 4.4 per cent; phosphorus, 8.9 per cent; sodium, 9.6 per cent; reducing sugar after hydrolysis, 45 per cent). In strains of staphylococci Julianelle & Wieghard (42) have demonstrated two type-specific carbohydrates. The specific polysaccharides of cholera and cholera-like vibrios are found by Linton & Mitra (43) to fall into three types, characterized by the nature of the constituent sugars and sugar acids. Bruce White

¹ For a review of the literature through 1934, see Mikulaszek (37).

(44) recognizes four specific carbohydrate-containing substances among the cholera vibrios. From defatted tubercle bacilli (BCG) Chargaff & Schaefer (45) have separated two serologically active polysaccharides, one being a water-soluble weak acid with mannose, *d*-arabinose, a small amount of inositol and 2.9 per cent of amino sugars, while the other is soluble in acids and has a large amount of firmly bound calcium phosphate. The recognition of the specific precipitable substance of *Brucella abortus* as a carbohydrate, reported by Favilli & Biancalani (46; cf. also 47), has been questioned by Hershey, Huddleson & Pennell (48) who attribute the specific reactions to a non-carbohydrate substance [cf. Schapira Biancalani (49)]. Castaneda (50; cf. also 51) has obtained two different preparations, apparently polysaccharides, from *Proteus* OX-19; the one which is alkali-stable appears to be responsible for the Weil-Felix reaction. Other papers deal with specific polysaccharides from gonococci (52), from *Saccharomyces cerevisiae* (53, 54), and other yeasts (54). The question of the presence of the same carbohydrate in rough and smooth strains of bacteria is considered by Bruce White (55). [A specific carbohydrate in *Ascaris lumbricoides* has been examined by Campbell (56).]

A general criticism concerning the studies on bacterial carbohydrates is made by Morgan (38). He points to the possibility of securing nonbacillary polysaccharides from agar-grown bacteria.

An examination of the literature reveals the frequency with which workers are content to designate their specific bacterial polysaccharide preparations "pure" provided they are protein-free and essentially polysaccharide in nature, while the presence of non-specific polysaccharides and serologically reactive agar-like substances is frequently overlooked . . .

In colorimetric determinations of the carbohydrate content of protein fractions of the scarlatinal streptococcus, Heidelberger & Kendall (57) obtained results from which is inferred the existence of carbohydrate-protein complexes.

While in the original work on bacterial polysaccharides principal concern was given to the isolation of protein-free preparations in order to prove the immunological significance of carbohydrates, there is at present an emphasis on using less drastic procedures designed to separate these substances in an unaltered state. Methods for obtaining pneumococcus polysaccharides have been worked out by Felton *et al.* (58), who utilize adsorption on calcium phosphate [$\text{Ca}_3(\text{PO}_4)_2$],

and by Brown (59) ; Hornus & Enders (60) report a new preparation of *Pneumococcus*-III specific polysaccharide (nitrogen, 0.3 per cent) which reacts with Type III (horse) antiserum after absorption with the original "SSS III" substance. Heidelberger and coworkers (61) have employed modifications in the methods for preparing the specific pneumococcus carbohydrates of Type I, II, and III, avoiding high temperatures, strong acid, or alkali [see also Chow (62)]. The substances differ from those previously described in giving considerably larger quantities of precipitate with homologous rabbit antisera and in yielding solutions characterized by much higher viscosity. The behavior of the new preparations, particularly their sensitivity to heat, is assumed to be due to greater molecular size of the material in the native state and the readiness with which they are partially depolymerized.

Concerning the question of the antigenicity of pure carbohydrates, brought into prominence by the observations on pneumococcus polysaccharides, Uhlenhuth & Remy (63) could not produce antibodies to glycogen or starches adsorbed on collodion, and they find that the antibodies formed upon injection of gum arabic are due to traces of proteins. The claim of antibody formation with starch has been similarly refuted [Fujimura (64)]. A specific polysaccharide from typhoid bacilli was reported to stimulate antibodies when injected in the form of an immune precipitate (65).

As with proteins, chemical alterations of polysaccharides produce changes in serological properties. Chow & Goebel (66) have shown that the reactivity of *Pneumococcus*-I polysaccharide is practically abolished on esterification with diazomethane, and is restored when the methyl ester is saponified. These observations are in keeping with previous results on the dominant influence of acid groups on specificity (34). A reduction in reactivity by methylation of hydroxyl groups in a *Pneumococcus*-III polysaccharide was observed by Heidelberger & Kendall (120*b*).

The synthesis of aldobionic acids for comparison with those found in bacterial carbohydrates has been initiated by Hotchkiss & Goebel (67) in the preparation of the β -heptaacetyl methyl ester of gentioburonic acid through the condensation of 1,2,3,4-tetraacetyl- β -glucose with 1-bromo-2,3,4-triacetylglucuronic acid methyl ester [Goebel & Babers (68)]. The compound is not identical with the corresponding derivative of the aldobionic acid of *Pneumococcus* Type III. In a similar way, galactose-6- β -glucuronide has been synthesized (67) and

found to be identical with the aldobionic acid from gum arabic, a material known to react with antisera for *Pneumococcus* II and III.

Blood group substances.—Subsequent to the reports by Freudenberg and coworkers [e.g., (69)] on a group-specific polysaccharide preparation from the urine of group-A human beings, carbohydrate preparations endowed with the group-A property have been secured from horse saliva [Landsteiner (70)] and from commercial pig pepsin and gastric mucin [Landsteiner & Chase (71)]; these all have a much higher serological activity than the substance from human urine (69) but like the latter contain galactose, aminohexose, and acetyl groups. In a recent note Freudenberg (72) mentions preparations from human urine and more active ones obtained from animal organs, which contain neither aminohexose nor uronic acid, in distinction to former results. As evidence for the carbohydrate nature of the A-substances, the destruction by certain polysaccharide-splitting bacteria has been offered (73; cf. also 74).

Carbohydrate-lipid complexes.—As regards non-protein antigens in bacteria, the antigenic complexes of carbohydrate and lipid described by Boivin, Mesrobianu and coworkers have been referred to before.² In tubercle bacilli (BCG strain), Chargaff & Schaefer (76) found an ether-soluble substance described as an "ester of a phosphorylated mannose-containing polysaccharide with palmitic acid and liquid saturated fatty acids of high molecular weight" (nitrogen, 0.4 per cent; phosphorus, 1.6 per cent). A polysaccharide complex from human tubercle bacilli is reported by Heidelberger & Menzel (77), who repeated their former work on these organisms and kept all solutions slightly acid to avoid possible alterations. One fraction indeed differed markedly from the former preparation, giving rise upon alkali treatment to a precipitate consisting mainly of magnesium palmitate; the polysaccharide recovered from the alkaline solution resembled that isolated by the original method. The existence of lipid-carbohydrate complexes in tubercle bacilli is questioned by Bloch (78).

Lipids.—Evidence for the presence in bacteria of serologically active lipids whose specificity is not due to carbohydrate groupings is brought by Macheboeuf and his colleagues (79). In the course of a methodical study of lipid fractions from *Myco. tuberculosis*, lipoidal material, possibly a single substance, has been separated (80); it con-

² *Ann. Rev. Biochem.*, 4, 571 (1935). For subsequent work, and the relation to bacillary endotoxins, see (75).

tains a hapten³ which gives specific complement-fixation. The preparation has practically no nitrogen, and yields no sugar on hydrolysis. The substance (phosphorus, 3.4 per cent) is soluble in ether and also in water. The statement that the lipid is the only serologically active hapten is in apparent contradiction to the results of others with polysaccharides [cf. (84)].

Several papers deal with attempts to isolate serologically active substances extracted from organs with lipid solvents. With regard to the Wassermann substance in beef-heart extracts, the reader is referred to papers by Oe. Fischer & Steinert (85), and O. Fischer & Günsberger (86). For the purification, Oe. Fischer makes use of the distribution of the active substance between aqueous alcohol and petroleum ether. The preparation of Fischer & Günsberger was obtained after removal of chloroform-soluble lipids; the activity of the final material was weak except when mixed with paraffin oil, fats, or phosphatides. Sakakibara (87) has reported that the main component responsible for the Wassermann reactivity is the optically inactive β -lecithin. Preparations of the Forssman substance from horse kidney, according to Brunius (88), exhibit a strong Molisch reaction and upon hydrolysis yield fatty acids; the preparations contain hexosamine, which may be a characteristic constituent of Forssman substances.⁴ Of considerable significance for the chemistry of the Forssman substance is the work of Morgan (38), mentioned above, on the isolation of an apparently pure bacterial polysaccharide (from *B. dysenteriae* Shiga) endowed with Forssman reactivity (137).

A second organ specific hapten in brain has been found by Sachs & Schwab (89) in the protagon fraction.

Regarding lipids of known chemical nature, Kimizuka (90) could find no antigenic action with purified egg lecithin. Wadsworth, Maltaner & Maltaner (91) cast doubt on the reports of the antigenicity of cholesterol. They consider the observed effects to be due to an increased anticomplementary property of the sera and point to differences between the reactions of true antibodies, and of the sera obtained after injection of cholesterol. The older view is upheld by Hahn &

³ Also see Sandor *et al.* (20, 81), and Pinner (82). The possibility of firm adsorption of carbohydrate on to lipid has been emphasized by Sandor [cf. Bloch (78, 83)].

⁴ A comprehensive and most valuable monograph by Brunius (88a) came into our hands too late for adequate treatment; the activity of the preparations varied with the glucosamine content.

Hazato (92; cf. also 93), who find that the anticomplementary properties of cholesterol antisera and their complement-fixation reactions do not run parallel. According to Hahn & Hazato, some of the peculiarities observed are to be found also with sera to other lipids. A final decision could be rendered by specificity tests with various sterols, which has not been done on a sufficiently extensive scale, perhaps because of difficulties in obtaining suitable sera. The production of antibodies by injection of lipoidal hormones along with serum has been reported by Brandt & Goldhammer (93a).

SYNTHETIC CONJUGATED ANTIGENS

In several papers artificial conjugated antigens have further been used for investigating general serological problems. From a study of the immunological properties of antigens containing aliphatic side chains [Landsteiner & van der Scheer (94)], it was seen that such substances, too, are capable of determining specificity; for instance, succinic acid can clearly be differentiated from malonic and glutaric acids. In inhibition tests with dibasic fatty acids, besides these specific reactions, others were observed which appeared to depend on the general physico-chemical properties of long aliphatic chains. The serological properties of some heterocyclic compounds have been investigated by Erlenmeyer & Berger (95). They studied the ability of various pyrazolone derivatives to inhibit the reaction between an azo-protein made from 4-amino-antipyrine and its immune serum. The structure common to those substances which inhibited seems to be $C_6H_5 \cdot N \cdot N \cdot CH_3$, which, the authors recall, is the group responsible for the pharmacological action of the drugs. The same investigators (96) prepared a serum against an antigen made from 3-aminopyridine for examining pyridine derivatives.⁵

In continuation of attempts to elucidate the specificity of native proteins with the aid of synthetic azoproteins made with peptide azo-components, Landsteiner & van der Scheer (97) have included antigens containing tetra- and penta-peptides. While precipitin tests with seventeen compounds built up from leucine and glycine showed cross reactions of varying strength between substances having the same terminal amino acid, inhibition tests with the corresponding nitro-benzoyl derivatives disclosed a high degree of specificity. These

⁵ The results were not confirmed in all respects upon repetition in our laboratory.

experiments, together with the demonstrated distinct specificity of azoproteins made from amino acids (98), would indicate that "a large number of serologically different peptides can be built up if one uses all the amino acids that occur in proteins."

In accord with results showing the prominent influence of acid groups on the specificity of azoproteins is the sharp serological differentiation between *p*-aminobenzyl- β -glucoside and *p*-aminobenzyl- β -glucuronide [Goebel (99)]. Very interesting is the observation that the glucuronic acid-protein antigen reacts in high dilution with horse (but not rabbit) immune sera prepared against pneumococci of Types II, III, or VIII, ascribable to the presence of glucuronic acid in the specific polysaccharides. However, the significance of parts of the specific carbohydrate molecule other than the uronic acid is revealed in that on absorption of pneumococcal immune serum (III) with the glucuronic acid antigen the resulting fluid still reacts strongly with *Pneumococcus* III or VIII polysaccharides. Along similar lines are experiments made by Woolf, Marrack & Downie (100), who studied derivatives of the natural glucuronide euxanthic acid as to reactivity with pneumococcus immune serum. Some of the compounds, e.g. aniline-azo-euxanthic acid, gave precipitates with Type II serum which could be inhibited by glucuronic acid, not by galacturonic or mannuronic acids. An azoprotein antigen prepared from amino-euxanthic acid was precipitated by Type II immune serum.

Haurowitz & Kraus (101) have examined anew the distribution of antigens after injection of conjugated antigens, and iodoglobulin; the antigen was found for the most part in the reticulo-endothelial system.

New antigens, specific by virtue of the introduced group, have been made by Gaunt, Higgins & Wormald (102) through the action of benzylcarbonyl chloride on native proteins.

ANTIBODIES

The generally accepted view of their protein nature is in evidence in the recent work on antibodies. The problem seems to reduce to that of ascertaining differences between the proteins of normal and immune sera, and in general the methods applied to the study of the antibodies follow closely those used in protein chemistry. The previous claims of Frankel & Olitsky, that by methods of adsorption and elution the typhoid agglutinin can be rendered protein-free, are re-

futed by Rosenheim (103). An extensive study has been made of the changes in the protein content of serum during immunization (104).

The progressive inactivation of antibodies by coupling with diazonium compounds was examined by Eagle, Smith & Vickers (105); with diphtheria antitoxin, the property of flocculating with toxin was destroyed earlier than its neutralizing capacity, and in antipneumococcus (horse) sera the reactivity with type-specific carbohydrate disappeared much more quickly than the agglutinating, protecting and complement-fixing properties. Eagle (106) has further confirmed previous reports (Eisler, and Smith & Marrack) that the attachment to diphtheria antitoxin, of toxin on the one hand and of antibodies against horse serum on the other, occurs at different sites on the molecule.

The type-specific antibody of *Meningococcus* Type I has been concentrated tenfold by Scherp & Rake (106*a*), who found it possible to precipitate the antibody completely by treatment with carbon dioxide subsequent to dialysis.

Chow & Goebel (66; cf. also 107) have described a new method employing phthalate buffer for the concentration of *Pneumococcus*-Type-I antibody from horse serum; it results in a solution of pseudoglobulin of which up to 90 per cent precipitates with Type-I-specific polysaccharide. The immune globulin precipitates at pH 7.6; the content of lysine was found to be slightly higher than in normal serum globulin. The preparation contained lipid (2.75 per cent) [see page 637] and gave a positive Molisch test. The nitrogen value was 14.85 per cent, lower than that for normal horse-serum globulin. Acetylation of the purified immune globulin with ketene, or treatment with formaldehyde, diminished markedly or abolished the reactivity; when the antibody was "deformolized," activity was again apparent. Another method for separating pneumococcus antibodies has been used by Heidelberger & Kendall (108). Based upon the finding that the reaction equilibrium is shifted by high salt concentrations, free antibody was shown to be liberated from immune precipitates upon extraction with 10 to 15 per cent sodium chloride solutions, 85 to 90 per cent of the protein thus recovered being reactive antibody. The yields in general varied between one-fifth and one-third of the total antibody content. The isoelectric points of such preparations were determined electrophoretically (118). Felton (109) notes that in 5 per cent urea solutions pneumococcus antibody is insoluble, unlike the proteins of normal horse serum, and is soluble in 40 per cent

solutions of urea. With pneumococcal polysaccharides Chow & Wu (110) have applied the method of recovery from specific antigen-antibody precipitates. Specific precipitins for azoproteins have been separated by absorption with insoluble antigens (stromata bearing the homologous azocomponent), followed by elution with dilute acetic acid (139). Furthermore, mention may be made of Goldie's studies on antibody purification (111) and absorption and elution experiments by Meyer & Pic (110a) with kaolin impregnated with antigen.

Neither the nitrogen partition nor the amount of carbohydrate enabled Hewitt (112) to differentiate between ordinary serum globulin and antitoxin; cystine and tyrosine were somewhat lower in the case of diphtheria toxin-antitoxin floccules. A careful analysis of specific precipitates of *Pneumococcus* I and II polysaccharides which may be considered to contain highly purified antibody proteins has been carried out by Calvery (113) with regard to nitrogen partition, sulfur, and amino acids.

The advances in the methods of ultrafiltration and ultracentrifugation furnished new means for the study of antibodies. Elford, Grabar & Fischer (114) upon filtering an old sample of antipneumococcus horse serum through collodion estimated that "75% of the antibody passed a 140 m μ membrane; rather less than 1% passed an 80 m μ membrane. . . ." The activity appeared to be associated with the largest globulins of the serum. In pursuing similar filtration experiments, Goodner, Horsfall & Bauer (115) found that "the smallest specific antibody of antipneumococcal rabbit-serum corresponds to a pore-size of 11 m μ , the smallest in horse-serum to a size of 44 m μ , while both horse- and rabbit-serum have large specific aggregates corresponding roughly to a pore-size of 88 m μ ." With a concentrated immune horse serum diluted 1:10 in broth the antibody was recovered between 150 and 188 m μ , but these large aggregates dispersed easily when dilution was made in normal serum. Biscoe, Herčík & Wyckoff (116), Wyckoff (117), and in Svedberg's laboratory, Heidelberger, Pedersen & Tiselius (118) have studied the concentration, in high gravitational fields, of the antibodies and the protein components of whole and concentrated antipneumococcal sera. The investigations of both groups with rabbit sera have shown no appreciable difference between the sedimentation constants of the globulin fraction of normal serum and immune sera or purified antibody solutions ($s = 7 \times 10^{-13}$ cm. sec.⁻¹ dyne⁻¹). But in the words of Heidelberger, "It is . . . clear that different fractions of the serum proteins carry

the antibody function in the horse and in the rabbit. It would thus appear that the rabbit produces antibody from the principal globulin component, while the horse develops pneumococcus I anticarbohydrate . . . from an otherwise minor component." With horse antiserum, the antibody activity was concentrated along with proteins having a sedimentation constant of about 16×10^{-13} , whereas the constant for the principal globulin of normal horse serum is 7.8×10^{-13} (Heidelberger).

In view of the similarity, with respect to their action on animal cells, of the plant hemagglutinins and antibodies, it is in place to mention that Sumner & Howell (119) describe a crystallized globulin (Concanavalin A) of the jack bean, which they identify with the agglutinin.

An hypothesis concerning the mechanism of antibody formation is considered by Stearn (119a).

REACTIONS BETWEEN ANTIGEN AND ANTIBODY

*Quantitative relationships.*⁶—Studies on quantitative relationships in the precipitin reaction have been continued by Heidelberger & Kendall with the method of determining nitrogen in the precipitates, and their theory of the reaction has been elaborated. Evidence is offered against the idea that non-antibody protein in the antiserum is incorporated in the precipitate (120a). (For the lipid content of specific precipitates see below.) The formation of the precipitate is ascribed to multivalency of both antibody and Pneumococcus-III polysaccharide and to the consequent ability of the various compounds formed to react with carbohydrate or antibody or with complexes of the two, so that "the reactions would lead to the formation of larger and larger aggregates until precipitation ultimately occurred," and the final precipitate would consist of molecules of antibody held together by polysaccharide molecules, similar to the idea proposed by Marrack. The precipitin reaction is interpreted on the basis of the mass law as being "the resultant of a series of competing bimolecular reactions, the quantitative outcome of which depends on the relative proportions in which the components are mixed." This theory is given mathematical formulation, and values derived from the formulae are concordant with the experimental findings for carbohydrate-anticar-

⁶ An instructive paper by Haurowitz (120) appeared after the manuscript was submitted.

bohydrate precipitation (120*b*), for an azoprotein antigen-antibody system (120*c*), for the reaction of crystalline egg albumin with its antibody (120*d*), and for agglutination, which is treated as a precipitin reaction at the surface (121). In the case of *Pneumococcus* Type I, Heidelberger & Kabat (122) have afforded quantitative proof of the identity of agglutinins and precipitins. Although their studies produce evidence that the antibody formed in response to a single, known antigen is not homogeneous (see p. 636), the authors state that the average behavior of the antibody is such that it can be treated mathematically as that of a single substance.

With regard to the pertinent question whether the equivalence point ratio is a constant for a given system, the values so far obtained by several investigators with the system ovalbumin-antiovalbumin agree fairly well, ranging on the whole between 10:1 and 11:1 (antibody nitrogen:antigen nitrogen), but somewhat higher and lower values have been observed [cf. (123)]. Heidelberger & Kendall (120*d*) present evidence to show that the ratio may vary with different antisera, and in different bleedings from one animal. On continued immunization the ratio became greater and the "equivalence zone," the region of complete precipitation of all antigen and antibody, became broader. For explanation the authors favor the idea that the "antibody formed in the later stages is reactive with an increased number of chemically distinct groupings" on the antigen molecule, a parallelism to the frequently observed broader range of cross reactions after longer immunization.

From a new point of view the theory underlying specific precipitation was taken up by Boyd & Hooker. Their assumption that the equivalence point represents the complete coating of the antigen molecule by molecules of antibody has been expressed in a formula designed to predict the ratio of antigen to antibody from the molecular weight of the antigen, the actual values indeed agreeing fairly well with this concept in the first data assembled. In a recent paper (124) dealing with proteins of high molecular weight, hemocyanins, the authors have found ratios significantly lower than those required by the formula, but considered to be of the right order of magnitude. The effect of the surface area of antigens in antibody reactions is discussed also by Merrill (125), who applied the relationship to viruses.

The formation of visible aggregates in the agglutinin or precipitin reactions has customarily been attributed to a non-specific flocculation by electrolytes following the "first stage" of serological reactions,

namely the invisible, specific union between antibodies and antigen. The view of Marrack, and of Heidelberger [cf. (120*b*)], that the precipitate consists of a formed lattice of "masses of antigen and antibody molecules bound together by specific linkages" (Topley) implies the action of specific forces also during the stage of aggregation. Evidence in favor of this concept was brought by Topley, Wilson & Duncan (126) who showed that when two kinds of bacteria and their homologous agglutinins were mixed, the resulting individual clumps were not indifferently composed of both the microorganisms but consisted of only one sort. That this phenomenon is not quite regular would follow from observations of Abramson (127) on mixed agglutination and Hooker (128) on mixed precipitin reactions.

In precipitin reactions where the method of optimal proportions has been used [cf. Brown (129)], the occurrence at times of more than one zone of particulation has been explained by the presence of a corresponding number of antigenic components. Indeed in antisera (to horse serum) which exhibited double zoning, Goldsworthy & Rudd (26) found both antiglobulin and antialbumin [cf. Taylor (130) and Hahn (131)]. But the finding of a single zone in a titration, as Taylor & Adair (132) demonstrate, is not necessarily indicative of a single antigen-antibody system. The speed of flocculation may be employed for quantitative studies, according to Hooker & Boyd (133; cf. also 128), for in the case of single antigens mixed with an excess of antibody there is a linear relation between concentration of antigen and the time for precipitation.

From quantitative relations found in the precipitation of diphtheria toxin by antitoxin [Healey & Pinfield (134)], it was concluded that the reactants, depending on the relative amounts present, can combine in two proportions, TA or TA_2 , where A is one flocculation unit of antitoxin and T is one Lf unit of toxin. The two sorts of precipitates could be interconverted: $TA + A \rightarrow TA_2$, or $TA_2 + T \rightarrow 2TA$. According to du Noüy & Hamon (135) the titration of diphtheria toxin and antitoxin can be made by finding the ratio giving the greatest change in viscosity, a viscometer affording continuous readings being used.

Cross reactions and absorption experiments.—The common observation that, by absorption with heterologous antigens, antibodies of different specificities can be demonstrated in individual immune sera has received attention in several papers: the exclusive validity of the concept of different antibodies as the reflection of an equal number

of substances in the immunizing antigen (the mosaic hypothesis) is questioned.⁷ Burnet (136), in examining the relationships among the Flexner dysentery bacilli by means of the reactions of bacteriophages, concludes that "while a given antiserum corresponding to a bacterial polysaccharide antigen can be readily fractionated by immunological methods into dissimilar parts, no such components can be demonstrated for the antigen." Evidence that different antibodies can be referred to two determinant groups in a single substance has been provided by Meyer & Morgan (137). If, as seems probable, their polysaccharide is homogeneous, its ability to combine with Shiga antisera of two sorts, one type chiefly heterophile (hemolytic) and the other exclusively antibacterial (agglutinating), would indicate that the substance "may contain in each of its molecules two kinds of determinant receptors of diverse specificity." A similar explanation for a case which probably is still more complex, the cross reactions of duck and hen ovalbumins, has been offered by Hooker & Boyd (138), each antigen in their opinion producing more than one kind of antibody by virtue of qualitatively different determinants within single molecules. On the other hand, it would follow from a study of azoprotein cross reactions by Landsteiner & van der Scheer (139) that a plurality of determinant groups in the antigen is not an absolute prerequisite for the formation of multiple antibodies. For example, antibodies produced in response to an azoprotein made from $m\text{-NH}_2 \cdot \text{C}_6\text{H}_4 \cdot \text{SO}_3\text{H}$, as demonstrated by the method of partial absorption with heterologous antigens giving cross reactions, are not identical, some having greater affinity for $m\text{-NH}_2 \cdot \text{C}_6\text{H}_4 \cdot \text{AsO}_3\text{H}_2$, others for $m\text{-NH}_2 \cdot \text{C}_6\text{H}_4 \cdot \text{COOH}$, and some for $o\text{-NH}_2 \cdot \text{C}_6\text{H}_4 \cdot \text{SO}_3\text{H}$, although all antibodies have maximal affinity for the homologous antigen. Since the immunizing antigen obviously does not have a plurality of chemical groupings which severally occur in the heterologous antigens, the conclusion appears warranted that antibodies formed in response to a single structure are not entirely uniform, but "vary to some extent around a main pattern."⁸ Likewise, Heidelberger & Kendall (120b) have assembled data suggesting that in

⁷ For a general discussion of the subject, see (34).

⁸ With some conjugated antigens having two distinct, serologically reactive groupings in the azocomponent, separate antibodies directed toward each grouping have been found (unpublished experiments of the senior author with van der Scheer).

Pneumococcus-III antibody solutions there are present more than one kind of antibodies combining with the homologous specific polysaccharide. Their evidence derives in part from the reactions of methylated polysaccharide, and in part from the study of quantitative relationships [see also (120*d*) and (108)].

That different precipitins in one serum exist as separate entities is indicated by the results of Hektoen & Welker (140), who found it possible after immunizing with a mixture of antigens to remove, electively, *in vivo*, any of the specific precipitins by injecting the corresponding homologous antigen, an effect previously shown *in vitro* (141). Similarly, Dean, Taylor & Adair (142) found that two specific precipitins developed by simultaneous immunization with two antigens react independently of one another. The converse, namely the reaction of single antibodies with two entirely different antigens as evidence for different binding groups in one antibody, is considered by Meyer (143; see also 144 and Wyckoff, 145).

Rôle of serum lipids.—As to the rôle of serum lipids in the reactions of antibodies, Horsfall & Goodner find that in Type-I-antipneumococcus sera, which lose their flocculating capacity wholly (horse antiserum) or in large part (rabbit antiserum) upon the extraction of lipids, this property is restored upon the addition of minute amounts of lecithin to extracted horse immune serum and of cephalin in the case of rabbit serum (146). Lipids, probably by reason of adsorption, are found to constitute from 4 to 51 per cent of the weight of washed specific precipitates, depending upon the lipid concentration in the antigen-antibody mixture. Lipid nitrogen, however, forms only a very small part, usually 1 to 4 per cent, of the total nitrogen of precipitates (147). Although antisera from the horse and rabbit have very similar "lipid patterns," the adsorption of phosphatide by the specific precipitates is selective, that formed from horse antibody appearing to be principally cephalin and that from immune rabbit serum almost wholly non-amino phosphatide, probably lecithin (148).

ALLERGY TO SIMPLE SUBSTANCES

Experimental skin sensitization in human beings has been achieved by Haxthausen (149) with metal salts; on account of the cross reactions observed (with nickel and cobalt) the author stresses the fact, amply demonstrated, that chemically related substances give overlapping immunological reactions. With mercuric chloride, chromium trioxide or formaldehyde, sensitization could be attained when the

substances were administered in mixture with foreign serum (150). Horsfall (151) has reported the sensitization of rabbits with formolized proteins.

With a number of simple compounds, positive and constant sensitization effects were obtained in guinea pigs by Landsteiner & Jacobs (152*a*) after repeated intracutaneous application of very small quantities of the substances, e.g., *p*-nitrosodimethylaniline and 1,2,4-chlorodinitrobenzene, the latter being a frequent excitant of allergy in industrial workers. Upon examination of a number of (chlor-, nitro-) substituted benzenes (152*b*), it appeared that only those substances are capable of sensitizing which possess an easily detachable substituent and form substitution compounds with bases. This supports the view that sensitization is due to the formation of conjugates in the animal and establishes a connection with the results previously obtained with conjugated antigens. In keeping with this conclusion, acyl chlorides (and benzyl chlorides) were found to sensitize the skin, and regularly to produce an anaphylactic state demonstrable by intravenous injection of the corresponding acylated protein. Also after intracutaneous sensitization with arsphenamine, lethal anaphylactic shock could be produced in a considerable number of the animals (152*c*; cf. also 153, 154).

Employing the same method as above, Nitti & Bovet (155) showed that, as in human beings, the new antistreptococcal drug, prontosil (the hydrochloride of 2,4-diaminoazobenzene-4'-sulfonamide), produced definite sensitization in guinea pigs.

Incidentally the result of Benjamins *et al* (156) may be mentioned, which suggests, in their opinion, that the active principles of pollen extracts are substances of low molecular weight which are activated by colloidal substances.

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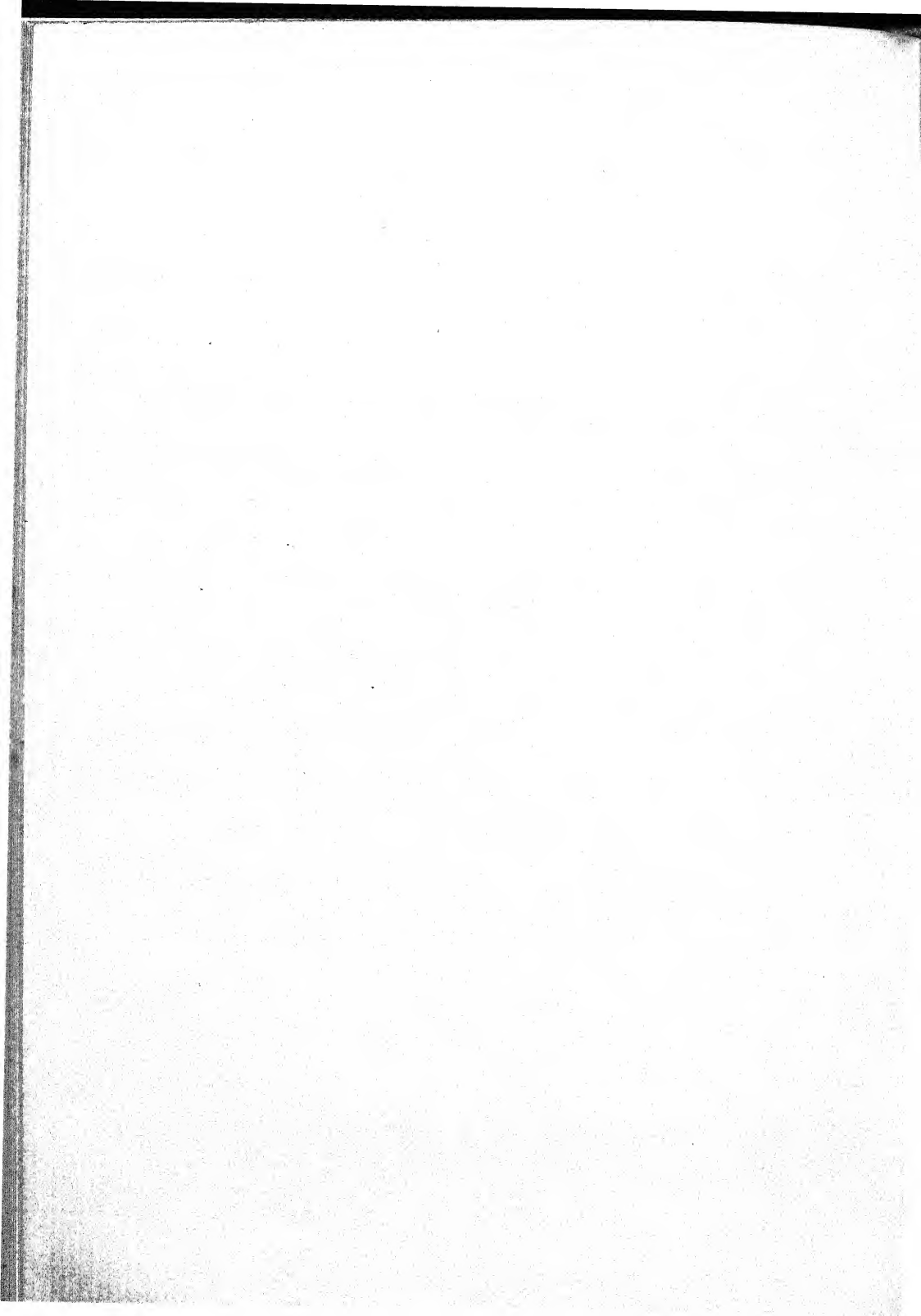
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